PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark Office, PCT
2011 South Clark Place Room
CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 07 November 2000 (07.11.00)	
International application No. PCT/CA00/00288	**Applicant's or agent's file reference** 3055-20/PAR
International filing date (day/month/year) 16 March 2000 (16.03.00)	**Priority date** (day/month/year) 17 March 1999 (17.03.99)
Applicant MISRA, Santosh et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

16 October 2000 (16.10.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Charlotte ENGER
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

Form PCT/IB/331 (July 1992)

CA0000288

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
3055-20/PAR		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/CA00/00288	16/03/2000	17/03/1999
International Patent Classification (IPC) or national classification and IPC C12N15/82		
Applicant University Of Victoria Innovation and Dev.....		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 8 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 2 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
16/10/2000	13.06.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Loubradou-Bourges, N Telephone No. +49 89 2399 7342

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/CA00/00288

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-5,7-37	as originally filed	
6	with telefax of	16/03/2001

Claims, No.:

1-13	as originally filed	
14	with telefax of	16/03/2001

Drawings, sheets:

1/3-3/3	as originally filed

Sequence listing part of the description, pages:

1-13, as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   - ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   - ☐ the language of publication of the international application (under Rule 48.3(b)).
   - ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   - ☒ contained in the international application in written form.
   - ☐ filed together with the international application in computer readable form.
   - ☐ furnished subsequently to this Authority in written form.
   - ☐ furnished subsequently to this Authority in computer readable form.
   - ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☒ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

☐ the description, pages:

☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☒ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)
see separate sheet

6. Additional observations, if necessary:
see separate sheet

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	3-12
	No:	Claims	1-2, 13
Inventive step (IS)	Yes:	Claims	12
	No:	Claims	1-11, 13
Industrial applicability (IA)	Yes:	Claims	1-13
	No:	Claims	

2. Citations and explanations
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Reference is made to the following documents:

D1: WO 99 06564 A (ECK JOYCE VAN ;BLOWERS ALAN D (US); SANFORD JOHN (US); SMITH FRANZ) 11 February 1999 (1999-02-11)
D2: WO 95 18855 A (PIONEER HI BRED INT) 13 July 1995 (1995-07-13)
D3: EP-A-0 552 559 (UNILEVER PLC ;UNILEVER NV (NL)) 28 July 1993 (1993-07-28)

Amendments filed with telefax of 16.03.01:

No basis for the subject-matter of the newly filed claim 14 could be founded in the description as originally filed. In particular, the passage p. 26. lines 6-19 cited by the applicant is considered not to provide support for said claim. Thus, the amendments filed introduces subject-matter which extends beyond the content of the application as filed, contrary to Art. 34 (2) (b).

SECTION V

The applicant has filed arguments with a telefax of 16.03.01 which were taken into account when drafting this IPER.

The present application relates to transgenic plants which are transformed with genes encoding anti-microbial peptides from the group of temporins or dermaseptins, and method of producing such peptides from the plant.

1. NOVELTY

The subject-matter of claims 1-2, 13 does not meet the requirements of Art. 33(2) PCT for the following reasons:

1.1 The IPEA understands that dermaseptins and temporins are distinct families recognized by those skilled in the art. However, the definition of temporins and dermaseptins as described in the present specification includes structurally

undefined variants (see p. 6-7 of the present application). The only limitation is their antimicrobial activity (see also Section VIII, point 1). Thus, the Magainin antimicrobial cationic peptide may be considered to represent a modified dermaseptin or temporin.
D1 discloses transgenic plants transformed with a Magainin gene which is expressed. Thus, D1 is prejudicial to the novelty of claims 1 and 2.
D1 (p.7 lines 23-27) also suggests the purification of magainin or magainin-like antimicrobial peptides from transgenic plant. Thus, the subject-matter of claim 13 is not novel.

1.2 Claims 2-12 relate to transgenic plants comprising specific sequence of dermaseptins or temporins, or recombinant nucleic acids which were not disclosed in the prior art, and are thus considered to be novel.

2. INVENTIVE ACTIVITY

The applicant has filed some arguments with respect to inventive step which are considered to be relevant for transgenic plant as defined in claim 12 but not to other or generic transgenic plants.
The subject-matter of claims 1-11, 13 does not meet the requirement of Art. 33(3) PCT for the following reasons:

2.1 The general problem underlying the present application is the provision of transgenic plants resistant to phytopathogens. The use of cationic peptides for this purpose is known (see D1 and D2).
D1, which is considered to represent the closest prior art, discloses transgenic plants resistant to microbial infection due to expressed antimicrobial genes, particularly Magainin and Magainin-related (PGL) peptide genes. D1 provides complete teaching for methods of transforming the plant tissue with a nucleic acid encoding antimicrobial magainin peptides, therefore leading to an improved resistance to phytopathogenic microbes.
In view of the prior art, the contribution of the present application is the provision of additional or further phytopathogen resistant plants. Because the use of antimicrobial cationic peptides in general and of magainins in particular in plant cell expression systems has already been described, it is not sufficient to merely suggest further subgroups of cationic peptides known to have antimicrobial effects

(namely subgroups of dermaseptins or temporins as defined in claim 1 or subgroups defined by SEQ ID as defined in claim 2). Rather, the claimed subgroups would have to distinguish themselves from the previously known contributions in an unexpected way. In the absence of a demonstrated unexpected effect associated with the use of dermaseptins or temporins defined as generic subgroups instead of magainins or any of the other previously used cationic peptides, the presently claimed plants are considered to represent but one of several possible further contributions, not involving inventive step. Therefore, the presence of an inventive activity in claims **1-2** cannot be acknowledged.

2.2 Dependent or independent claims **3-11** do not add any features which meet the requirement of PCT with respect to inventive step, for the following reasons:
- In the absence of any new and unexpected effect provided by the particular amino acid sequences shown in SEQ ID N°3-14 (encoding various dermaseptins) referred to in claims 3-5, 8-9, and in SEQ ID N°17-26 (encoding various temporins) referred to in claims 3-5, 10-11, the disclosure of transgenic plants expressing said particular sequences does not involve an inventive activity.
- The addition of a N terminal peptide extension either naturally occurring or synthetic as described in claims 4-5 or of an "anionic pro-region", insofar as said term may be understood (see Section VIII point 1.3) as described in claims 6-7, 9, 11 is a matter of normal design procedure to a skilled person in the art.

2.3 For the reasons mentioned in point 1, the subject-matter of claim **13** is also not inventive.

2.4 The graph Shown in Fig.1 provides a basis for the alleged effects of transgenic plants expressing dermaseptin B or temporin A on resistance to soft rot. The applicant has also furnished some arguments with the telefax of reply to the written opinion unexpected effect which are suitable to establish the presence of inventive activity in claim 12 in particular in comparison with transgenic potato resistant to soft rot due the expression of magainin as described in the prior art (see D3). Thus, claim **12** is considered to be inventive.

SECTION VIII

1. The present application does not meet the requirement of Art. 6 PCT since claims 1-11, 13 are not clear for the following reasons:

1.1. The terms "temporins" and "dermaseptins" are open definitions although said terms are employed in the prior art, insofar as said terms refer to the unclear expressions "fragments" and "variants" of naturally occurring peptides (see p. 6-7 of the present application) and as they are only limitated by a "dermaseptin" or "temporin" activity which also appear to be unclear (see the comments below). Said terms should be defined by technical features. In addition, the definition of the terms "temporins" and "dermaseptins" refers to a biological activity which is merely an antimicrobial activity. Since the application fails to provide test for screening for a activity specific to said compounds, references to such activities is unclear and does not provide a distinguishable feature. Correspondingly, claims 1-2, 7 and dependent claims 3-5 referring to the unclear terms "dermaseptins" or "temporins", and claims 8, 10 and dependent claims 9, 11 referring to a biological activity, are unclear.

1.2 The expression "biologically active cationic peptide" (claim 13) is unduly broad and open to interpretation, and do not appear to be limited to any activity specific to dermaseptins or temporins.

1.3 The expression "fragment" of a amino acid sequence (referred to in claims 8, 10) is unclear as it may encompass any individual amino acid insofar as the definition of a biological activity which should defined and restrict the scope claimed is unclear.

1.4 Claims 1-11 are considered to be not supported by the description since the application only provides transgenic plants comprising an amino acid sequence selected from the group of SEQ ID N° 28 and 34, and fails to provide any technical support for generalization to the whole scope claimed, namely transgenic plants comprising others dermaseptins or temporins, since the provision of further transgenic plants comprising and expressing active cationic peptide is not routine matter, as admitted by the applicant itself.

The same reasoning applies to the method of producing a biologically active cationic peptide as defined in claim 13.

SEQ IDs: 33 shows the nucleic acid sequence encoding $MSRA_3$.

SEQ ID: 34 shows the amino acid sequence of $MSRA_3$.

SEQ IDs: 35-38 show the oligos used to generate the nucleic acid sequence encoding $MSRA_3$.

SEQ IDs: 39-41 show the amino acid sequences of various N-terminal extension sequences.

Brief Description of the Figures

Figure 1 is a graph that shows the results from assays that tested the resistance of transgenic potato tubers to soft rot. Discs prepared from tubers of *Desiree* control and transgenic plants expressing Demaseptin B (sample Nos. D1, D2, D6, D10) or Temporin A (sample Nos. T1, T2, T3) were infected with *E. carotovora* (black boxes) or left uninfected (white boxes). After 6 days at RT, rotted tissue was gently removed from the discs and the sensitivity/resistance to *E. carotovora* was expressed as the loss of weight of tuber tissue.

Figure 2 is a graph that shows the bactericidal effect of the peptides $MSRA_2$ (Dermaseptin B) and $MSRA_3$ (Temporin A) on *E. coli*. The cell cultures were incubated at room temperature in the presence of indicated concentration of Dermaseptin B (DSB; 7 μg/ml, 30 μg/ml, and 75 μg/ml), Temporin A (TA; 75 μg/ml, 133 μg/ml, 200 μg/ml) and a combination of Temporin A and Dermaseptin B (133 μg/ml Temporin A and 30 μg/ml Dermaseptin B) for 4 hours, diluted and plated on LB plates. After overnight incubation at 37°C, the colonies were counted and the survival of bacteria was scored.

Figure 3 is a graph that shows the bactericidal effect of the peptides $MSRA_2$ (Dermaseptin B) and $MSRA_3$ (Temporin A) on *E. carotovora*. The cell cultures were incubated at room temperature in the presence of indicated concentration of Dermaseptin B (DSB; 23 μg/ml, 45 μg/ml) or Temporin A (TA; 67 μg/ml, 133 μg/ml) for 4 hours, diluted and plated on LB plates. After overnight incubation at 28°C, the colonies were counted and the survival of bacteria was scored.

I. Definitions

Dermas ptin: As used herein, the term "dermaseptin" refers to any member of the family of naturally occurring cationic peptides term d d rmaseptins,

11. A transgenic plant according to claim 8 wherein the peptide further comprises an anionic pro-region peptide operably linked to the N-terminus of the peptide.

12. A transgenic plant comprising a recombinant nucleic acid molecule encoding a peptide comprising an amino acid sequence selected from the group consisting of SEQ IDs: 28 and 34.

13. A method of producing a biologically active cationic peptide comprising:

providing a transgenic plant according to claim 1; and

isolating at least one biologically active cationic peptide from the plant.

14. The method of claim 13, wherein the cationic peptide is selected from the group consisting of the dermaseptins set forth in SEQ ID NOs 3-14, and the temporins set forth in SEQ ID NOs 17-26.

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

Rae, Patricia, A.
Sim & McBurney
330 University Avenue
6th Floor
Suite 600
Toronto, Ontario M5G 1R7
CANADA

NOTIFICATION OF TRANSMITTAL OF
THE INTERNATIONAL PRELIMINARY
EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)*	13.06.2001
Applicant's or agent's file reference: 3055-20/PAR	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/CA00/00288	16/03/2000	17/03/1999

Applicant: University Of Victoria Innovation and Dev.....

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Zoglauer, H Tel.+49 89 2399-8051

Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
3055-20/PAR		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/CA00/00288	16/03/2000	17/03/1999
International Patent Classification (IPC) or national classification and IPC C12N15/82		
Applicant University Of Victoria Innovation and Dev.....		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 8 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 2 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
16/10/2000	13.06.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Loubradou-Bourges, N Telephone No. +49 89 2399 7342

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/CA00/00288

I. Basis f th r p rt

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-5,7-37	as originally filed	
6	with telefax of	16/03/2001

Claims, No.:

1-13	as originally filed	
14	with telefax of	16/03/2001

Drawings, sheets:

1/3-3/3	as originally filed

Sequence listing part of the description, pages:

1-13, as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   - ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   - ☐ the language of publication of the international application (under Rule 48.3(b)).
   - ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   - ☒ contained in the international application in written form.
   - ☐ filed together with the international application in computer readable form.
   - ☐ furnished subsequently to this Authority in written form.
   - ☐ furnished subsequently to this Authority in computer readable form.
   - ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☒ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

☐ the description, pages:

☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☒ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)
see separate sheet

6. Additional observations, if necessary:
see separate sheet

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	3-12
	No:	Claims	1-2, 13
Inventive step (IS)	Yes:	Claims	12
	No:	Claims	1-11, 13
Industrial applicability (IA)	Yes:	Claims	1-13
	No:	Claims	

2. Citations and explanations
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Reference is made to the following documents:

D1: WO 99 06564 A (ECK JOYCE VAN ;BLOWERS ALAN D (US); SANFORD JOHN (US); SMITH FRANZ) 11 February 1999 (1999-02-11)
D2: WO 95 18855 A (PIONEER HI BRED INT) 13 July 1995 (1995-07-13)
D3: EP-A-0 552 559 (UNILEVER PLC ;UNILEVER NV (NL)) 28 July 1993 (1993-07-28)

Amendments filed with telefax of 16.03.01:

No basis for the subject-matter of the newly filed claim 14 could be founded in the description as originally filed. In particular, the passage p. 26. lines 6-19 cited by the applicant is considered not to provide support for said claim. Thus, the amendments filed introduces subject-matter which extends beyond the content of the application as filed, contrary to Art. 34 (2) (b).

SECTION V

The applicant has filed arguments with a telefax of 16.03.01 which were taken into account when drafting this IPER.

The present application relates to transgenic plants which are transformed with genes encoding anti-microbial peptides from the group of temporins or dermaseptins, and method of producing such peptides from the plant.

1. NOVELTY

The subject-matter of claims 1-2, 13 does not meet the requirements of Art. 33(2) PCT for the following reasons:

1.1 The IPEA understands that dermaseptins and temporins are distinct families recognized by those skilled in the art. However, the definition of temporins and dermaseptins as described in the present specification includes structurally

undefined variants (see p. 6-7 of the present application). The only limitation is their antimicrobial activity (see also Section VIII, point 1). Thus, the Magainin antimicrobial cationic peptide may be considered to represent a modified dermaseptin or temporin.
D1 discloses transgenic plants transformed with a Magainin gene which is expressed. Thus, D1 is prejudicial to the novelty of claims 1 and 2.
D1 (p.7 lines 23-27) also suggests the purification of magainin or magainin-like antimicrobial peptides from transgenic plant. Thus, the subject-matter of claim 13 is not novel.

1.2 Claims 2-12 relate to transgenic plants comprising specific sequence of dermaseptins or temporins, or recombinant nucleic acids which were not disclosed in the prior art, and are thus considered to be novel.

2. INVENTIVE ACTIVITY
The applicant has filed some arguments with respect to inventive step which are considered to be relevant for transgenic plant as defined in claim 12 but not to other or generic transgenic plants.
The subject-matter of claims 1-11, 13 does not meet the requirement of Art. 33(3) PCT for the following reasons:

2.1 The general problem underlying the present application is the provision of transgenic plants resistant to phytopathogens. The use of cationic peptides for this purpose is known (see D1 and D2).
D1, which is considered to represent the closest prior art, discloses transgenic plants resistant to microbial infection due to expressed antimicrobial genes, particularly Magainin and Magainin-related (PGL) peptide genes. D1 provides complete teaching for methods of transforming the plant tissue with a nucleic acid encoding antimicrobial magainin peptides, therefore leading to an improved resistance to phytopathogenic microbes.
In view of the prior art, the contribution of the present application is the provision of additional or further phytopathogen resistant plants. Because the use of antimicrobial cationic peptides in general and of magainins in particular in plant cell expression systems has already been described, it is not sufficient to merely suggest further subgroups of cationic peptides known to have antimicrobial effects

(namely subgroups of dermaseptins or temporins as defined in claim 1 or subgroups defined by SEQ ID as defined in claim 2). Rather, the claimed subgroups would have to distinguish themselves from the previously known contributions in an unexpected way. In the absence of a demonstrated unexpected effect associated with the use of dermaseptins or temporins defined as generic subgroups instead of magainins or any of the other previously used cationic peptides, the presently claimed plants are considered to represent but one of several possible further contributions, not involving inventive step. Therefore, the presence of an inventive activity in claims **1-2** cannot be acknowledged.

2.2 Dependent or independent claims **3-11** do not add any features which meet the requirement of PCT with respect to inventive step, for the following reasons:
- In the absence of any new and unexpected effect provided by the particular amino acid sequences shown in SEQ ID N°3-14 (encoding various dermaseptins) referred to in claims 3-5, 8-9, and in SEQ ID N°17-26 (encoding various temporins) referred to in claims 3-5, 10-11, the disclosure of transgenic plants expressing said particular sequences does not involve an inventive activity.
- The addition of a N terminal peptide extension either naturally occurring or synthetic as described in claims 4-5 or of an "anionic pro-region", insofar as said term may be understood (see Section VIII point 1.3) as described in claims 6-7, 9, 11 is a matter of normal design procedure to a skilled person in the art.

2.3 For the reasons mentioned in point 1, the subject-matter of claim **13** is also not inventive.

2.4 The graph Shown in Fig.1 provides a basis for the alleged effects of transgenic plants expressing dermaseptin B or temporin A on resistance to soft rot. The applicant has also furnished some arguments with the telefax of reply to the written opinion unexpected effect which are suitable to establish the presence of inventive activity in claim 12 in particular in comparison with transgenic potato resistant to soft rot due the expression of magainin as described in the prior art (see D3). Thus, claim **12** is considered to be inventive.

SECTION VIII

1. The present application does not meet the requirement of Art. 6 PCT since claims 1-11, 13 are not clear for the following reasons:

1.1. The terms "temporins" and "dermaseptins" are open definitions although said terms are employed in the prior art, insofar as said terms refer to the unclear expressions "fragments" and "variants" of naturally occurring peptides (see p. 6-7 of the present application) and as they are only limitated by a "dermaseptin" or "temporin" activity which also appear to be unclear (see the comments below). Said terms should be defined by technical features. In addition, the definition of the terms "temporins" and "dermaseptins" refers to a biological activity which is merely an antimicrobial activity. Since the application fails to provide test for screening for a activity specific to said compounds, references to such activities is unclear and does not provide a distinguishable feature. Correspondingly, claims 1-2, 7 and dependent claims 3-5 referring to the unclear terms "dermaseptins" or "temporins", and claims 8, 10 and dependent claims 9, 11 referring to a biological activity, are unclear.

1.2 The expression "biologically active cationic peptide" (claim 13) is unduly broad and open to interpretation, and do not appear to be limited to any activity specific to dermaseptins or temporins.

1.3 The expression "fragment" of a amino acid sequence (referred to in claims 8, 10) is unclear as it may encompass any individual amino acid insofar as the definition of a biological activity which should defined and restrict the scope claimed is unclear.

1.4 Claims 1-11 are considered to be not supported by the description since the application only provides transgenic plants comprising an amino acid sequence selected from the group of SEQ ID N° 28 and 34, and fails to provide any technical support for generalization to the whole scope claimed, namely transgenic plants comprising others dermaseptins or temporins, since the provision of further transgenic plants comprising and expressing active cationic peptide is not routine matter, as admitted by the applicant itself.

The same reasoning applies to the method of producing a biologically active cationic peptide as defined in claim 13.

SEQ IDs: 33 shows the nucleic acid sequence encoding $MSRA_3$.

SEQ ID: 34 shows the amino acid sequence of $MSRA_3$.

SEQ IDs: 35-38 show the oligos used to generate the nucleic acid sequence encoding $MSRA_3$.

SEQ IDs: 39-41 show the amino acid sequences of various N-terminal extension sequences.

Brief Description of the Figures

Figure 1 is a graph that shows the results from assays that tested the resistance of transgenic potato tubers to soft rot. Discs prepared from tubers of *Desiree* control and transgenic plants expressing Demaseptin B (sample Nos. D1, D2, D6, D10) or Temporin A (sample Nos. T1, T2, T3) were infected with *E. carotovora* (black boxes) or left uninfected (white boxes). After 6 days at RT, rotted tissue was gently removed from the discs and the sensitivity/resistance to *E. carotovora* was expressed as the loss of weight of tuber tissue.

Figure 2 is a graph that shows the bactericidal effect of the peptides $MSRA_2$ (Dermaseptin B) and $MSRA_3$ (Temporin A) on *E. coli*. The cell cultures were incubated at room temperature in the presence of indicated concentration of Dermaseptin B (DSB; 7 μg/ml, 30 μg/ml, and 75 μg/ml), Temporin A (TA; 75 μg/ml, 133 μg/ml, 200 μg/ml) and a combination of Temporin A and Dermaseptin B (133 μg/ml Temporin A and 30 μg/ml Dermaseptin B) for 4 hours, diluted and plated on LB plates. After overnight incubation at 37°C, the colonies were counted and the survival of bacteria was scored.

Figure 3 is a graph that shows the bactericidal effect of the peptides $MSRA_2$ (Dermaseptin B) and $MSRA_3$ (Temporin A) on *E. carotovora*. The cell cultures were incubated at room temperature in the presence of indicated concentration of Dermaseptin B (DSB; 23 μg/ml, 45 μg/ml) or Temporin A (TA; 67 μg/ml, 133 μg/ml) for 4 hours, diluted and plated on LB plates. After overnight incubation at 28°C, the colonies were counted and the survival of bacteria was scored.

I. Definitions

Dermaseptin: As used herein, the term "dermaseptin" refers to any member of the family of naturally occurring cationic peptides termed dermaseptins,

11. A transgenic plant according to claim 8 wherein the peptide further comprises an anionic pro-region peptide operably linked to the N-terminus of the peptide.

12. A transgenic plant comprising a recombinant nucleic acid molecule encoding a peptide comprising an amino acid sequence selected from the group consisting of SEQ IDs: 28 and 34.

13. A method of producing a biologically active cationic peptide comprising:

providing a transgenic plant according to claim 1; and

isolating at least one biologically active cationic peptide from the plant.

14. The method of claim 13, wherein the cationic peptide is selected from the group consisting of the dermaseptins set forth in SEQ ID NOs 3-14, and the temporins set forth in SEQ ID NOs 17-26.

PATENT COOPERATION TREATY

From the:
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

Rae, Patricia, A.
Sim & McBurney
330 University Avenue
6th Floor
Suite 600
Toronto, Ontario M5G 1R7
CANADA

PCT

WRITTEN OPINION

(PCT Rule 66)

Date of mailing *(day/month/year)*	19.12.2000
Applicant's or agent's file reference: 3055-20/PAR	**REPLY DUE** **within 3 month(s)** from the above date of mailing

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/CA00/00288	16/03/2000	17/03/1999

International Patent Classification (IPC) or both national classification and IPC
C12N15/82

Applicant
University Of Victoria Innovation and Dev.....

1. This written opinion is the **first** drawn up by this International Preliminary Examining Authority.

2. This opinion contains indications relating to the following items:

I	☒	Basis of the opinion
II	☐	Priority
III	☐	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
IV	☐	Lack of unity of invention
V	☒	Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
VI	☐	Certain document cited
VII	☐	Certain defects in the international application
VIII	☒	Certain observations on the international application

3. The applicant is hereby **invited to reply** to this opinion.

When? See the time limit indicated above. The applicant may, before the expiration of that time limit, request this Authority to grant an extension, see Rule 66.2(d).

How? By submitting a written reply, accompanied, where appropriate, by amendments, according to Rule 66.3. For the form and the language of the amendments, see Rules 66.8 and 66.9.

Also: For an additional opportunity to submit amendments, see Rule 66.4.
For the examiner's obligation to consider amendments and/or arguments, see Rule 66.4 bis.
For an informal communication with the examiner, see Rule 66.6.

If no reply is filed, the international preliminary examination report will be established on the basis of this opinion.

4. The final date by which the international preliminary examination report must be established according to Rule 69.2 is: 17/07/2001.

Name and mailing address of the international preliminary examining authority:
European Patent Office
D-80298 Munich
Tel. +49 89 2399 - 0 Tx: 523656 epmu d
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Authorized officer / Examiner
Loubradou-Bourges, N

Formalities officer (incl. extension of time limits)
Emslander, S
Telephone No. +49 89 2399 8718

I. Basis of th pini n

1. This opinion has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed".*):

Description, pages:

1-37 as originally filed

Claims, No.:

1-13 as originally filed

Drawings, sheets:

1/3-3/3 as originally filed

Sequence listing part of the description, pages:

1-13, as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☒ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☒ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   ☐ the description, pages:
   ☐ the claims, Nos.:
   ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrialapplicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	1-2, 13
Inventive step (IS)	Claims	1-13
Industrial applicability (IA)	Claims	

2. Citations and explanations
   see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Reference is made to the following documents:

D1: WO 99 06564 A (ECK JOYCE VAN ;BLOWERS ALAN D (US); SANFORD JOHN (US); SMITH FRANZ) 11 February 1999 (1999-02-11)
D2: WO 95 18855 A (PIONEER HI BRED INT) 13 July 1995 (1995-07-13)
D3: EP-A-0 552 559 (UNILEVER PLC ;UNILEVER NV (NL)) 28 July 1993 (1993-07-28)

SECTION V

The present application relates to transgenic plants which are transformed with genes encoding anti-microbial peptides from the group of temporins or dermaseptins, and method of producing such peptides from the plant.

1. The subject-matter of claims 1-2, 13 does not meet the requirements of Art. 33(2) and (3) PCT for the following reasons:

   The definition of temporins and dermaseptins includes structurally undefined variants (see p. 6-7 of the present application). The only limitation is their antimicrobial activity (see also Section VIII, point 1). Thus, the Magainin antimicrobial cationic peptide may be considered to represent a modified dermaseptin or temporin.
   D1 discloses transgenic plants transformed with a Magainin gene which is expressed. Thus, D1 is prejudicial to the novelty of claims 1 and 2.
   D1 (p.7 lines 23-27) also suggests the purification of magainin or magainin-like antimicrobial peptides from transgenic plant. Thus, the subject-matter of claim 13 is not novel.

2. The subject-matter of claims 1-13 does not meet the requirement of Art. 33(3) PCT for the following reasons:

2.1 The general problem underlying the present application is the provision of transgenic plants resistant to phytopathogens. The use of cationic peptides for this purpose is known (see D1 and D2).
D1, which is considered to represent the closest prior art, discloses transgenic plants resistant to microbial infection due to expressed antimicrobial genes, particularly Magainin and Magainin-related (PGL) peptide genes. D1 provides complete teaching for methods of transforming the plant tissue with a nucleic acid encoding antimicrobial magainin peptides, therefore leading to an improved resistance to phytopathogenic microbes.
In view of the prior art, the contribution of the present application is the provision of additional or further phytopathogen resistant plants. Because the use of antimicrobial cationic peptides in general and of magainins in particular in plant cell expression systems has already been described, it is not sufficient to merely suggest further subgroups of cationic peptides known to have antimicrobial effects. Rather, the claimed subgroups would have to distinguish themselves from the previously known contributions in an unexpected way. In the absence of a demonstrated unexpected effect associated with the use of dermaseptins or temporins instead of magainins or any of the other previously used cationic peptides, the presently claimed plants are considered to represent but one of several possible further contributions, not involving inventive step. Therefore, the presence of an inventive activity in claims 1-2 cannot be acknowledged.

2.2 Dependent or independent claims 3-11 do not add any features which meet the requirement of PCT with respect to inventive step, for the following reasons:
- In the absence of any new and unexpected effect provided by the particular amino acid sequences shown in SEQ ID N°3-14 (encoding various dermaseptins) referred to in claims 3-5, 8-9, and in SEQ ID N°17-26 (encoding various temporins) referred to in claims 3-5, 10-11, the disclosure of transgenic plants expressing said particular sequences does not involve an inventive activity.
- The addition of a N terminal peptide extension either naturally occurring or synthetic as described in claims 4-5 or of an "anionic pro-region", insofar as said term may be understood (see Section VIII point 1.3) as described in claims 6-7, 9, 11 is a matter of normal design procedure to a skilled person in the art.

2.3 For the reasons mentioned in point 1, the subject-matter of claim 13 is also not

inventive.

2.4 Insofar as the graph Shown in Fig.1 may be understood (see Section VIII, point 2), said graph provides a basis for the alleged effects of transgenic plants expressing dermaseptin B or temporin A on resistance to soft rot. Nevertheless, no comparative data showing a new or unexpected effect of the transgenic plants as claimed in claim 12 in comparison with transgenic potato resistant to soft rot due the expression of magainin as described in the prior art (see D3) are provided. Thus, the presence of an inventive activity in claim 12 cannot be acknowledged.

SECTION VIII

1. The present application does not meet the requirement of Art. 6 PCT since claims 1-11, 13 are not clear for the following reasons:

1.1. The terms "temporins" and "dermaseptins" are open definitions, insofar as said terms refer to the unclear expressions "fragments" and "variants" of naturally occurring peptides (see p. 6-7 of the present application). Said terms should be defined by technical features. In addition, the definition of the terms "temporins" and "dermaseptins" refers to a biological activity which is merely an antimicrobial activity. Since the application fails to provide test for screening for a activity specific to said compounds, references to such activities is unclear and does not provide a distinguishable feature. Correspondingly, claims 1-2, 7 and dependent claims 3-5 referring to the unclear terms "dermaseptins" or "temporins", and claims 8, 10 and dependent claims 9, 11 referring to a biological activity, are unclear.

1.2 The expression "biologically active cationic peptide" (claim 13) is open to interpretation.

1.3 The expression "anionic pro-region peptide" (referred to in claims 6-7, 9-11) and "spacer peptide" (referred to in claim 7) are vague and unclear.

1.4 The expression "fragment" of a amino acid sequence (referred to in claims 8, 10)

is unclear as it may encompass any individual amino acid.

1.5 Claims 1-11, 13 are considered to be not supported by the description since the application only provides transgenic plants comprising an amino acid sequence selected from the group of SEQ ID N° 28 and 34, and fails to provide any technical support for generalization to the whole scope claimed.

2. The Figure n°1 is not understandable since the white and black boxes are not defined. Correspondingly, the results of the resistance test as described in Example 6 p.32-33 cannot be clearly interpreted. The applicant is invited to add a legend defining boxes as for example non infected/infected, as could be deducted from the description.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

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(54) Title: TRANSGENIC PLANTS THAT ARE RESISTANT TO A BROAD SPECTRUM OF PATHOGENS

(57) Abstract

Transgenic plants that express dermaseptin and/or temporin peptides are disclosed. In certain embodiments, these plants have enhanced, broad–spectrum pathogen resistance and are useful as agricultural or horticultural crops. In other embodiments, the plants are used to produce large quantities of the dermaseptin and/or temporin peptides.

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TRANSGENIC PLANTS THAT ARE RESISTANT TO A BROAD SPECTRUM OF PATHOGENS

FIELD OF THE INVENTION

This invention relates to plants that are genetically engineered to express one or more peptides belonging to the temporin and/or dermaseptin families.

BACKGROUND OF THE INVENTION

Plants are hosts to thousands of infectious diseases caused by a vast array of phytopathogenic fungi, bacteria, viruses, and nematodes. These pathogens are responsible for significant crop losses worldwide, resulting from both infection of growing plants and destruction of harvested crops. The most widely practiced methods of reducing the damage caused by such pathogens involve the use of various chemical agents. Unfortunately, many pathogens develop resistance to such chemicals, and some pathogens (especially viruses) are not susceptible to control by chemical means. In addition, many of the chemical agents used are broad-spectrum toxins, and may cause serious environmental damage, as well as toxicity in humans.

Plant breeding and, more recently, genetic engineering techniques have also been employed to combat plant pathogens. In certain instances, breeders and molecular biologists have successfully engineered resistance to certain pathogens. In the last few years, a number of plant *R* (resistance) genes have been isolated from plants. When introduced into otherwise susceptible crops, these *R* genes produce enhanced resistance to certain pathogens. For example, U.S. patent No. 5,571,706 describes the isolation of the tobacco *N* gene, which confers enhanced resistance to Tobacco Mosaic Virus. However, while conventional breeding and genetic engineering approaches reported to date can successfully enhance pathogen resistance, they typically address problems caused by just one pathogen, or a small number of closely related pathogens. As a result, while crops produced using these approaches may have enhanced protection against one pathogen, conventional chemical agents must still be used to control others.

It would be of great agricultural benefit to be able to produce plants having enhanced resistance to a broad spectrum of pathogens, including bacterial and fungal pathogens. It is to such plants that the present invention is directed.

SUMMARY OF THE INVENTION

The present inventors have discovered that the expression of certain peptides in transgenic plants confers broad spectrum pathogen resistance, including enhanced resistance to both fungal and bacterial pathogens. The peptides in question are small, positively charged (cationic) peptides belonging to the temporin and dermaseptin families, which occur naturally in the skin of certain species of frog. Transgenic plants provided by the invention may be used in conventional agricultural applications, such as food crops. Alternatively, the plants may be harvested and processed to extract the expressed temporin and/or dermaseptin peptides, which may then be purified for use in medical and other applications.

The invention thus encompasses transgenic plants that express at least one dermaseptin or temporin peptide, and methods of making such plants. Parts of such plants, including seeds, fruit, stems, leaves and roots, may be utilized in conventional ways as food sources, or as a source of the dermaseptin or temporin peptides. Because all plant types are susceptible to one or more plant pathogens, the present invention may be usefully applied to produce broad-spectrum resistance in any plant type. Thus, the invention may be applied to both monocotyledonous, dicotyledenous and gymnosperm plants, including, but not limited to maize, wheat, rice, barley, soybean, cotton, beans in general, rape/canola, alfalfa, flax, sunflower, safflower, brassica, cotton, flax, peanut, clover; vegetables such as lettuce, tomato, cucurbits, cassava, potato, carrot, radish, pea, lentils, cabbage, cauliflower, broccoli, Brussels sprouts, peppers; tree fruits such as citrus, apples, pears, peaches, apricots, walnuts; and flowers such as orchides, carnations and roses.

In its most basic form, the invention provides transgenic plants that express one or more dermaseptin and/or temporin peptides. Members of the dermaseptin and temporin peptide families are well known in the art. Examples of dermaseptins that may be used in the invention include, but are not limited to, the dermaseptins described by Mor. et al., *Biochemistry*, **30**:8824-8830, 1991, Strahilevitz, *Biochemistry*, **33**:10951-

10960, 1994 and Wechselberger, *Biochim. Biophys. Acta* **1388**: 279-283, 1998. Examples of temporins that may be used include, but are not limited to, the temporins described by Simmaco et al., *Eur. J. Biochem.*, **242**:788-92, 1996. In their natural state (i.e., expressed in frog cells), both dermaseptin and temporin peptides are produced as precursor forms that are subsequently processed by proteolytic cleavage to form mature proteins. The mature forms of dermaseptins are typically about 27-34 amino acids in length, while the mature forms of temporins are typically about 10-13 amino acids in length. The invention contemplates the use of both the naturally occurring full-length (unprocessed) forms of these peptides, as well as the mature (processed) forms of the peptides and intermediate forms. In addition, synthetic forms of the peptides may also be employed. Synthetic forms of the peptides include any form that is not naturally occurring, and encompasses peptides that differ in amino acid sequence from the naturally occurring peptides, but which still retain dermaseptin or temporin biological activity. Such sequence variants will typically retain at least 40% amino acid sequence identity with at least one naturally occurring dermaseptin or temporin peptide.

Other synthetic forms of dermaseptins and temporins that may be employed include forms having N-terminal peptide extensions. Such peptide extensions may comprise portions of the precursor forms of dermaseptins or temporins that are usually removed during protein processing, or may be synthetic sequences. These N-terminal peptide extensions may serve to provide enhanced resistance to proteolytic cleavage, and may also enhance the antimicrobial activity of the peptides. Typically, these N-terminal extensions are of between 2 and 25 amino acids in length, although longer extensions may also be employed. Examples of N-terminal extension sequences that are utilized in certain embodiments include the peptide sequences MAMWK and MASRH. The AMWK sequence is a naturally-occurring peptide extension; it is part of the full-length dermaseptin-b peptide sequence that is normally cleaved during processing. The ASRH is a synthetic extension sequence. In each case, the N-terminal methionine is added to the extension peptide to ensure proper expression of the peptide.

While the fundamental aspect of the invention is based on the expression of temporin and dermaseptin peptides in transgenic plants, other amino acid sequences may be joined to the peptides in order to produce fusion peptides. Expression of such fusion peptides in transgenic plants may provide even more effective broad-spectrum pathogen

resistance than expression of temporin or dermaseptin peptides alone, or may enhance stability of the expressed dermaseptin/temporin molecule to provide higher expression levels, and thereby facilitate purification of the peptide from plant tissues. Thus, in other embodiments, the invention provides transgenic plants that express a fusion peptide comprising:

(1) a first peptide sequence that is a dermaseptin or a temporin; and

(2) a second peptide sequence operably linked to the first peptide sequence.

The second peptide sequence is typically, but not necessarily, linked to the amino (N-) terminus of the first peptide sequence.

In certain embodiments, the second peptide sequence comprises an anionic (negatively charged) "pro-region" peptide sequence. Such pro-region peptides serve to neutralize the cationic nature of the dermaseptin or temporin and may thus provide enhanced stability in the cellular environment. Thus, these pro-regions generally include a number of negatively charged amino acids, such as glutamate (Glu or E) and aspartate (Asp or D). Suitable pro-regions include those that are found in naturally occurring unprocessed (full-length) dermaseptin and temporin peptides, as well as anionic pro-regions from other peptides, including those of mammalian origin, such as the pro-region from sheep cathelin proteins. Fusion peptides that include such pro-regions may be represented as P-D or P-T, wherein P is the pro-region peptide, T is a temporin peptide and D is a dermaseptin peptide.

Although such pro-region peptides may be directly joined to the N-terminus of the dermaseptin or temporin peptide, it may be beneficial to join the two peptides using a spacer peptide. The use of spacer peptides to join two peptide domains is well known in the art; such spacer peptides are typically of between 2 and 25 amino acids in length, and provide a flexible hinge connecting the first peptide sequence to the second peptide. Spacer sequences that have been used to provide flexible hinges connecting two peptide sequences include the glycine(4) serine spacer (GGGGS x3) described by Chaudhary et al., *Nature* **339:** 394-397, 1989. Alternatively, an N-terminal peptide extension as described above may serve to provide the spacer peptide function. Fusion peptides that comprise a pro-region peptide, a spacer peptide and a dermaseptin or temporin peptide may be represented as P-S-D or P-S-T, wherein S represents the spacer peptide.

Spacer sequences may also include a cleavage site, such as a peptide sequence recognized and cleaved by a protease. Such sites facilitate removal of the pro-region from the dermaseptin or temporin peptide following purification from plant tissues.

These and other aspects of the invention are described in more detail in the following sections.

SEQUENCE LISTING

The nucleic and amino acid sequences listed in the accompanying sequence listing are shown using standard letter abbreviations for nucleotide bases, and three letter code for amino acids. Only one strand of each nucleic acid sequence is shown, but the complementary strand is understood as included by any reference to the displayed strand.

SEQ ID: 1 shows the dermaseptin b cDNA sequence.

SEQ ID: 2 shows the amino acid sequence of the precursor (unprocessed) dermaseptin b peptide.

SEQ ID: 3 shows the 27 amino acid sequence of the mature dermaseptin b peptide.

SEQ ID: 4 shows the 31 amino acid sequence of the mature dermaseptin B peptide.

SEQ IDs: 5-14 show the amino acid sequences of various mature (processed) dermaseptin peptides.

SEQ ID: 15 shows a cDNA sequence encoding temporin G.

SEQ ID: 16 shows the amino acid sequence of the precursor (unprocessed) form of temporin G.

SEQ ID: 17 shows the 13 amino acid sequence of the mature temporin G peptide.

SEQ IDs: 18-26 show the amino acid sequences of various mature (processed) temporin peptides.

SEQ ID: 27 shows the nucleic acid sequence encoding $MSRA_2$.

SEQ ID: 28 shows the amino acid sequence of $MSRA_2$.

SEQ IDs: 29-32 show the oligos used to generate the nucleic acid sequence encoding $MSRA_2$.

SEQ IDs: 33 shows the nucleic acid sequence encoding $MSRA_3$.

SEQ ID: 34 shows the amino acid sequence of $MSRA_3$.

SEQ IDs: 35-38 show the oligos used to generate the nucleic acid sequence encoding $MSRA_3$.

SEQ IDs: 39-41 show the amino acid sequences of various N-terminal extension sequences.

Brief Description of the Figures

Figure 1 is a graph that shows the results from assays that tested the resistance of transgenic potato tubers to soft rot. Discs prepared from tubers of *Desiree* control and transgenic plants expressing Demaseptin B (sample Nos. D1, D2, D6, D10) or Temporin A (sample Nos. T1, T2, T3) were infected with *E. carotovora*. After 6 days at room temperature, rotted tissue was gently removed from the discs and the sensitivity/resistance to *E. carotovora* was expressed as the loss of weight of tuber tissue.

Figure 2 is a graph that shows the bactericidal effect of the peptides $MSRA_2$ (Dermaseptin B) and $MSRA_3$ (Temporin A) on *E. coli*. The cell cultures were incubated at room temperature in the presence of indicated concentration of Dermaseptin B (DSB; 7 μg/ml, 30 μg/ml, and 75 μg/ml), Temporin A (TA; 75 μg/ml, 133 μg/ml, 200 μg/ml) and a combination of Temporin A and Dermaseptin B (133 μg/ml Temporin A and 30 μg/ml Dermaseptin B) for 4 hours, diluted and plated on LB plates. After overnight incubation at 37°C, the colonies were counted and the survival of bacteria was scored.

Figure 3 is a graph that shows the bactericidal effect of the peptides $MSRA_2$ (Dermaseptin B) and $MSRA_3$ (Temporin A) on *E. carotovora*. The cell cultures were incubated at room temperature in the presence of indicated concentration of Dermaseptin B (DSB; 23 μg/ml, 45 μg/ml) or Temporin A (TA; 67 μg/ml, 133 μg/ml) for 4 hours, diluted and plated on LB plates. After overnight incubation at 28°C, the colonies were counted and the survival of bacteria was scored.

I. Definitions

Dermaseptin: As used herein, the term "dermaseptin" refers to any member of the family of naturally occurring cationic peptides termed dermaseptins, (Strahilevitz, *Biochemistry*, **33**:10951-960, 1994) as well as fragments and variants of

these naturally occurring peptides that display dermaseptin biological activity as defined below.

Dermaseptins were first identified in skin extracts from the South American arboreal frog *Phyllomedusa sauvagii* (Mor et al., *J. Biol. Chem.*, **269**: 31635-31641, 1994). They are broad-spectrum microbiocidal peptides that inhibit growth of filamentous fungi as well as bacteria, yeast, and protozoa (Strahilevitz, *Biochemistry*, **33**:10951-10960, 1994). Since the first dermaseptin, dermaseptin S, was identified, a number of other members of this peptide family have been characterized and cloned, including: dermaseptin-b, isolated from the skin of *Phyllomedusa bicolor* (Mor et al., *J. Biol. Chem.*, **269**: 31635-31641, 1994). (SEQ ID: 2); two dermaseptins isolated from *Pachymedusa dacnicolor*, and encoded by clones PD-3-3 and PD-2-2, as described by Wechselberger, *Biochim. Biophys. Acta* **1388**:279-283, 1998 (the peptide sequences of which are shown in SEQ IDs: 5-6, respectively); three dermaseptins isolated from *Agalychnis annae* and encoded by clones AA-3-6, AA-3-3, AA-3-1, as described by Wechselberger, *Biochim. Biophys. Acta* **1388**:279-283, 1998 (the peptide sequences of which are shown in SEQ IDs: 7-9, respectively); and five dermaseptin peptides from *Phyllomedusa sauvagii*, termed dermaseptin 5, dermaseptin 4, dermaseptin 3, dermaseptin 2, and dermaseptin 1, as described by Mor and Nicolas, *Journal Biochemical Chemistry*, **269**:1934-1939, 1994 (the peptide sequences of which are provided in SEQ IDs: 10-14, respectively). These sequences are readily available from public databases, including from GenBank.

Dermaseptin peptides are typically expressed as precursor forms of around 60-80 amino acids in length, and are subsequently processed to mature forms of around 27-34 amino acids in length. For example, the cDNA encoding dermaseptin-b (SEQ ID:1; Amiche et al., *J. Biol. Chem.* **269:**1747-1852, 1994; Chapentier et al., *Biol. Chem.* **273:**14690-14697, 1998; located in the GenBank nucleotide sequence database under accession number X72387) encodes a precursor peptide of 78 amino acids in length (SEQ ID: 2). This precursor form of dermaseptin-b is processed to produce two mature forms, termed dermaseptin b and dermaseptin B (Strahilevitz, *Biochemistry*, **33**:10951-10960, 1994). Dermaseptin b (SEQ ID: 3) is 27 amino acids in length and comprises amino acid residues 49-75 of the precursor form. Dermaseptin B is an alternative cleavage product of 31 amino acids in length, and

includes an N terminal extension of 4 amino acids (AMWK) (SEQ ID: 4). Dermaseptin B comprises amino acid residue 45-75 of the precursor form. With the exception of SEQ IDs: 1 and 2 which show the full–length precursor form of dermaseptin b, the dermaseptin peptides shown in the Sequence Listing represent the processed, mature forms of the peptides.

Given the availability of a wide range of dermaseptin peptide sequences, and the nucleic acid sequences that encode these peptides, one of ordinary skill in the art will readily be able to produce these peptides, and their corresponding nucleic acid sequences, using standard molecular biology techniques.

In addition to the use of the naturally occurring dermaseptin peptides described above, it will be apparent to one of skill in the art that the invention may be practiced using peptides that vary somewhat from the naturally occurring dermaseptin peptides, yet which nevertheless confer enhanced broad spectrum pathogen resistance when expressed in plants. For example, the N-terminal ∀-helical amphipathic segment of the mature dermaseptin peptides, particularly the first 18 amino acid residues, has been identified as important for antimicrobial activity (Mor et al., *J. Biol. Chem.*, **269**:31635-31641, 1994; Mor and Nicolas, *Journal Biochemical Chemistry*, **269**:1934-39, 1994) and this fragment may be used in place of the full-length mature dermaseptin. Thus, the term "dermaseptin" also encompasses variant dermaseptin peptides, as well as fragments of the naturally occurring peptides, that share a specified level of sequence identity with a naturally occurring dermaseptin peptide, or that differ from a naturally occurring dermaseptin peptide by one or more conservative amino acid substitutions.

Such variant peptides and fragments retain dermaseptin biological activity, which may be assayed by the methods described below. A variant dermaseptin will typically share at least 40% amino acid sequence identity with a naturally occurring dermaseptin peptide (such as the one shown in SEQ ID: 3) as determined by the methods described below.

Dermaseptin biological activity: the ability of a dermaseptin peptide to inhibit bacterial growth and/or fungal growth. Dermaseptin biological activity can readily be ascertained by using the protocols given below.

The antibacterial activity of a given dermaseptin peptide is assessed by determining its ability to inhibit the growth of a pectinolytic bacterial strain such as *Erwinia carotovora* or *Escherichia coli* DH5∀. The activity of a given peptide is determined by serially diluting the peptide in LB and aliquoting 100 :l to wells in a 96 well microtiter plate. A fresh bacterial culture (~0.3 A550) is then grown on Luria-Bertani medium (LB) (1% w/v tryptone and 0.5% w/v yeast extract) and diluted to 10^{-2} in LB to represent approximately $10^4 - 10^5$ colony forming units (CFU) ml^{-1}. 10 :l of the bacterial culture is then inoculated into the wells containing the peptide and the samples are incubated at 37 °C for 4 hours. The well contents are then diluted in LB, plated on LB agar and incubated overnight at 37 °C. The colonies on plates corresponding to each dilution of dermaseptin (and a control to which no peptide was added) are then counted, and the antibacterial activity of the peptide under test is determined by comparison to the control plate.

The dermaseptin peptide is determined to have biological activity if, under the conditions of this assay, it is capable of inhibiting bacterial growth by at least 10% at a concentration of 7 :g per ml (i.e., at this concentration, the number of bacterial colonies is no more than 90% that of the control plate).

The antifungal activity of a given dermaseptin peptide is assessed by utilizing the fungal strains *Phytophthora cactorum* and/or *Fusarium solani*. The selected fungal strain is grown on Five Cereal Agar (FCA containing 20 gL^{-1} five cereal baby food instant flakes, and 8 gL^{-1} agar[3] (Terras et al., *The Plant Cell* 7:573-588, 1995). After 5 days growth at room temperature a mycelial plug is removed and placed upside down in the center of a fresh FCA plate. A sterile solution (10 :l) of the test peptide is then introduced into a well 3 cm from the edge of the plate and a control well containing sterile water is established on the same plate. Various concentrations of the test peptide may be tested on the same plate, or on other plates. The assay plates incubated for 5 days at room temperature, after which the zone of growth inhibition around each well is measured.

The dermaseptin peptide is determined to have biological activity if, under the conditions of this assay, it is capable of inhibiting fungal growth at a concentration of

5 :g per ml (i.e., there is a discernible zone of inhibition around a well containing this concentration of peptide).

Temporin: As used herein, the term "temporin" refers to any member of the family of naturally occurring cationic peptides termed temporins (Simmaco et al., *Eur. J. Biochem.*, **242**:788-92. 1996) as well as fragments and variants of these naturally occurring peptides that display temporin biological activity as defined below.

Temporins are small cationic peptides with anti-microbial activity that were initially identified from a cDNA library prepared from the skin of the frog, *Rana temporaria*. These peptides show some sequence similarity to hemolytic peptides isolated from *Vespa* venom, however, the temporin peptides are not hemolytic (Simmaco et al., *Eur. J. Biochem.*, **242**:788-92. 1996).

Ten members of the temporin family, temporins A, B, C, D, E, F, G, H, K, and L, have been described by Simmaco et al., *Eur. J. Biochem.*, **242**:788-92. 1996. Like dermaseptins, temporins are typically expressed in a precursor form and subsequently processed to produce a mature form. For example, the cDNA molecule that encodes temporin G (shown in SEQ ID: 15, and located in the GenBank nucleotide database under accession number Y09395) encodes a 61 amino acid precursor form of temporin G (shown in SEQ ID: 16). Amino acids 1-22 comprise a signal sequence, amino acids 23-46 comprise a pro-region, and amino acids 47-59 comprise the processed, mature temporin G peptide (the mature form is shown in SEQ ID: 17). In general, the predicted mature temporin peptides are between 10 and 13 amino acids long, and some have been found to be amidated at the C-terminus (Simmaco et al., *Eur. J. Biochem.*, **242**:788-92. 1996). The mature forms of temporins A, B, C, D, E, F, G, H, K, and L are shown in SEQ IDs: 18, 19, 20, 21, 22, 23, 17, 24, 25, and 26, respectively.

Given the availability of a wide range of temporin peptide sequences, and the nucleic acid sequences that encode these peptides, one of ordinary skill in the art will readily be able to produce these peptides, and their corresponding nucleic acid sequences, using standard molecular biology techniques.

In addition to the use of the naturally occurring temporin peptides described above, it will be apparent to one of skill in the art that the invention may be practiced

using peptides that vary somewhat from the naturally occurring temporin peptides, yet which nevertheless confer enhanced broad spectrum pathogen resistance when expressed in plants. Thus, the term "temporin" also encompasses variant temporin peptides, as well as fragments of the naturally occurring peptides, that share a specified level of sequence identity with a naturally occurring temporin peptide, or that differ from a naturally occurring temporin peptide by one or more conservative amino acid substitutions.

Such variant peptides and fragments retain temporin biological activity, which may be assayed by the methods described below. A variant temporin will typically share at least 40% amino acid sequence identity with a naturally occurring temporin peptide (such as the one shown in SEQ ID: 17) as determined by the methods described below.

Temporin biological activity: the ability of a temporin peptide to inhibit bacterial growth.

The antibacterial activity of a given temporin peptide is assessed by determining its ability to inhibit the growth of a pectinolytic bacterial strain such as *Erwinia carotovora* or *Escherichia coli* DH5∀. The activity of a given peptide is determined by serially diluting the peptide in LB and aliquoting 100 :l to wells in a 96 well microtiter plate. A fresh bacterial culture (~0.3 A550) is then grown on Luria-Bertani medium (LB) (1% w/v tryptone and 0.5% w/v yeast extract) and diluted to 10^{-2} in LB to represent approximately $10^4 - 10^5$ colony forming units (CFU) ml^{-1}. 10 :l of the bacterial culture is then inoculated into the wells containing the peptide and the samples are incubated at 37°C for 4 hours. The well contents are then diluted in LB, plated on LB agar and incubated overnight at 37°C. The colonies on plates corresponding to each dilution of temporin (and a control to which no peptide was added) are then counted, and the antibacterial activity of the peptide under test is determined by comparison to the control plate.

The temporin peptide is determined to have biological activity if, under the conditions of this assay, it is capable of inhibiting bacterial growth by at least 10% at a concentration of 100 :g per ml (i.e., at this concentration, the number of bacterial colonies is no more than 90% that of the control plate).

The antifungal activity of a given temporin peptide is assessed by utilizing the fungal strains *Phytophthora cactorum* and/or *Fusarium solani*. The selected fungal strain is grown on Five Cereal Agar (FCA containing 20 gL^{-1} five cereal baby food instant flakes, and 8 gL^{-1} agar[3] (Terras et al., *The Plant Cell*, 7:573-588, 1995). After 5 days growth at room temperature a mycelial plug is removed and placed upside down in the center of a fresh FCA plate. A sterile solution (10 :l) of the test peptide is then introduced into a well 3 cm from the edge of the plate and a control well containing sterile water is established on the same plate. Various concentrations of the test peptide may be tested on the same plate, or on other plates. The assay plates incubated for 5 days at room temperature, after which the zone of growth inhibition around each well is measured.

The temporin peptide is determined to have biological activity if, under the conditions of this assay, it is capable of inhibiting fungal growth at a concentration of 5 :g per ml (i.e., there is a discernible zone of inhibition around a well containing this concentration of peptide).

Transgenic plant: As used herein, this term refers to a plant that contains recombinant genetic material not normally found in a wild-type plant of this species. Thus, a plant that is grown from a plant cell into which recombinant DNA is introduced by transformation is a transgenic plant, as are all offspring of that plant that contain the introduced transgene (whether produced sexually or asexually).

Sequence identity: The similarity between two nucleic acid sequences, or two amino acid sequences is expressed in terms of the level of sequence identity shared between the sequences. Sequence identity is typically expressed in terms of percentage identity; the higher the percentage, the more similar the two sequences are.

Methods of alignment of sequences for comparison are well known in the art. Various programs and alignment algorithms are described in: Smith and Waterman, *Adv. Appl. Math*, 2:482, 1981; Needleman and Wunsch, *J. Mol. Biol.*, **48**:443, 1970; Pearson and Lipman, *Proc. Natl. Acad. Sci. USA*, **85**:2444, 1988; Higgins & Sharp,*Gene*, **73**:237-244, 1988; Higgins & Sharp, *CABIOS*, 5:151-153, 1989; Corpet et al., *Nucleic Acids Research*, **16**:10881-10890, 1988; Huang, et al., *Computer Applications in the Biosciences*, **8**:155-165, 1992; and Pearson et al., *Methods in Molecular Biology*, **24**:307-331, 1994. Altschul et al., *Nature Gene* **6**:119-129, 1994

presents a detailed consideration of sequence alignment methods and homology calculations.

The NCBI Basic Local Alignment Search Tool (BLAST) (Altschul et al., *J. Mol. Biol.* **215**:403-410, 1990) is available from several sources, including the National Center for Biotechnology Information (NCBI, Bethesda, MD) and on the Internet, for use in connection with the sequence analysis programs blastp, blastn, blastx, tblastn and tblastx. It can be accessed at http//www.ncbi.nlm.nih.gov/BLAST/. A description of how to determine sequence identity using this program is available at http://www.ncbi.nlm.nih.gov/BLAST/blast_help.html.

Variants of naturally occurring dermaseptin and temporin peptides useful in the present invention are typically characterized by possession of at least 40% sequence identity counted over the full-length alignment with the amino acid sequence of a naturally occurring temporin or dermaseptin peptide when aligned using the NCBI Blast 2.0.1 (described in Altschul et al., *Nucleic Acids Res.* **25**:3389-3402, 1997). For comparisons of amino acid sequences of greater than about 30 amino acids, the Blast 2 sequences function is employed using the default BLOSUM62 matrix set to default parameters, (gap existence cost of 11, and a per residue gap cost of 1). When aligning short peptides (fewer than around 30 amino acids), the alignment should be performed using the Blast 2 sequences function, employing the PAM30 matrix set to default parameters (open gap 9, extension gap 1 penalties). Proteins with even greater similarity to the reference sequences will show increasing percentage identities when assessed by this method, such as at least 45%, at least 50%, at least 70%, at least 80%, at least 85%, at least 90%, or at least 95% sequence identity.

Recombinant: A recombinant nucleic acid is one that has a sequence that is not naturally occurring or has a sequence that is made by an artificial combination of two otherwise separated segments of sequence. This artificial combination is often accomplished by chemical synthesis or, more commonly, by the artificial manipulation of isolated segments of nucleic acids, e.g., by genetic engineering techniques.

Olig nucleotide (lig): A linear polynucleotide sequence of up to about 100 nucleotide bases in length.

Probes and primers: Nucleic acid probes and primers may readily be prepared based on the amino acid sequence provided by this invention. A probe comprises an isolated nucleic acid attached to a detectable label or reporter molecule. Typical labels include radioactive isotopes, ligands, chemiluminescent agents, and enzymes. Methods for labeling and guidance in the choice of labels appropriate for various purposes are discussed, e.g., in Sambrook et al., *Molecular Cloning: A Laboratory Manual, Cold Spring Harbor,* 1989 and Ausubel et al., *Current Protocals in Molecular Biology,* Greene Publishing Associates and Wiley-Intersciences, 1987.

Primers are short nucleic acids, preferably DNA oligonucleotides 15 nucleotides or more in length. Primers may be annealed to a complementary target DNA strand by nucleic acid hybridization to form a hybrid between the primer and the target DNA strand, and then extended along the target DNA strand by a DNA polymerase enzyme. Primer pairs can be used for amplification of a nucleic acid sequence, e.g., by the polymerase chain reaction (PCR) or other nucleic-acid amplification methods known in the art.

Methods for preparing and using probes and primers are described, for example, in Sambrook et al., *Molecular Cloning: A Laboratory Manual, Cold Spring Harbor,* 1989; Ausubel et al., *Current Protocals in Molecular Biology,* Greene Publishing Associates and Wiley-Intersciences, 1987; and Innis et al., *PCR Protocols, A Guide to Methods and Applications,* 1990. PCR primer pairs can be derived from a known sequence, for example, by using computer programs intended for that purpose such as Primer (Version 0.5, © 1991, Whitehead Institute for Biomedical Research, Cambridge, MA). One of skill in the art will appreciate that the specificity of a particular probe or primer increases with its length. Thus, for example, a primer comprising 20 consecutive nucleotides will anneal to a target with a higher specificity than a corresponding primer of only 15 nucleotides. Thus, in order to obtain greater specificity, probes and primers may be selected that comprise 20, 25, 30, 35, 40, 50 or more consecutive nucleotides.

Isolated: An "isolated" biological component (such as a nucleic acid or protein or organelle) has been substantially separated or purified away from other

biological components in the cell of the organism in which the component naturally occurs, i.e., other chromosomal and extra-chromosomal DNA and RNA, proteins and organelles. Nucleic acids and proteins that have been "isolated" include nucleic acids and proteins purified by standard purification methods. The term also embraces nucleic acids and proteins prepared by recombinant expression in a host cell as well as chemically synthesized nucleic acids.

Vector: A nucleic acid molecule as introduced into a host cell, thereby producing a transformed host cell. A vector may include nucleic acid sequences that permit it to replicate in a host cell, such as an origin of replication. A vector may also include one or more selectable marker genes and other genetic elements known in the art.

Operably linked: A first nucleic acid sequence is operably linked with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter affects the transcription or expression of the coding sequence. Generally, operably linked DNA sequences are contiguous and, where necessary to join two protein-coding regions, in the same reading frame. Two peptide sequences may be operably linked through a normal peptide bond, or by other covalent linkage.

II. Selection of Dermaseptin and Temporin Peptides

a. Dermaseptin Peptides

A listing of exemplary dermaseptin peptides is provided above. Nucleic acid molecules encoding these dermaseptin polypeptides may either be derived by simple application of the genetic code to the peptide sequence, or the naturally occurring cDNA or gene sequence may be employed. For example, a cDNA sequence encoding dermaseptin-b is provided in SEQ ID: 1 (and disclosed in Amiche et al., *J. Biol. Chem.* 269:1747-852, 1994). Typically, the mature form of the dermaseptin peptide will be selected for expression. However, any fragment of a full-length dermaseptin peptide may be selected, contingent upon that fragment having dermaseptin biological activity if it is to be used to produce pathogen-resisting plants.

One of ordinary skill in the art will appreciate that the various dermaseptin peptides have varying degrees of anti-microbial activity, with some working more effectively against certain pathogens then others. Therefore, when selecting peptides for producing transgenic plants with enhanced pathogen resistance, the selection of a particular dermaseptin will be depend upon, among other factors, the type of plant in which the peptide is to be expressed, and the types of pathogens that commonly infect that plant type.

Having selected the desired dermaseptin peptide to be expressed, a nucleic acid molecule encoding the peptide may be produced by standard molecular biology techniques. Because the dermaseptin peptides are relatively short, the simplest way to synthesize the nucleic acid molecule will likely be via synthesis of overlapping oligonucleotides on a commercially available oligonucleotide synthesizer. The oligonucleotides can then be assembled into a full-length coding region in vitro. This approach also permits the selection of codons encoding particular amino acid residues that reflect the codon usage bias of the plant into which the nucleic acid molecule is to be introduced, thereby enhancing the expression efficiency. Detailed examples of the production of coding sequences using this approach are provided in the examples below.

b. Temporin Peptides

A listing of exemplary temporin peptides is provided above. Nucleic acid molecules encoding these temporin peptides may either be derived by simple application of the genetic code to the peptide sequence, or the naturally occurring cDNA or gene sequence may be employed. For example, the cDNA sequence encoding temporin G is provided in SEQ ID: 15. Typically, the mature form of the temporin peptide will be selected for expression. However, any fragment of a full-length temporin peptide may be selected, contingent upon that fragment having dermaseptin biological activity if it is to be used to produce pathogen-resisting plants.

As with the selection of dermaseptin peptides, one of ordinary skill in the art will appreciate that the various temporin peptides have varying degrees of anti-microbial activity, with some working more effectively against certain pathogens then others. Therefore, when selecting peptides for producing transgenic plants with enhanced pathogen resistance, the selection of a particular temporin will be depend

upon, among other factors, the type of plant in which the peptide is to be expressed, and the types of pathogens that cause losses in that plant type.

As described above for dermaseptins, the synthesis and assembly of overlapping oligonucleotides is a simple and convenient way to produce nucleic acid molecules that encode temporins.

c. Addition of Other Peptide Sequences

The temporin and dermaseptin proteins may be also expressed in transgenic plants in the form of fusion proteins. Although any desired peptide may be fused to the selected dermaseptin or temporin protein for expression in plants, the expression of fusion proteins comprising an anionic pro-region peptide operably linked to the amino terminus of the dermaseptin or temporin is expected to be particularly beneficial. Any anionic pro-region peptide may be employed for this purpose, including the anionic pro-regions that are found in naturally occurring full-length (i.e., unprocessed) dermaseptin and temporin peptides. For example, the pro-region comprising amino acids 23-46 of temporin G (shown in SEQ ID: 16) may be used as a pro-region. Such pro-region peptides serve to neutralize the cationic nature of the dermaseptin or temporin and may thus provide enhanced stability in the cellular environment. Thus, these pro-regions generally include a number of negatively charged amino acids, such as glutamate (Glu or E) and aspartate (Asp or D).

Examples of other naturally occurring pro-region peptides that are known in the art include pro-region peptides of the following proteins: the human neutrophil defensin protein (Daher et al., *Proc. Natl. Acad. Sci USA*, **85**:7327-7331, 1988); the bovine antimicrobial cathelicidin protein BMAP28 (Skerlavaj et al., *J. Biol. Chem.* **271**: 28375-381, 1996); the sheep antimicrobial cathelin family of proteins (Mahoney et al., *FEBS Lett.* **377**:519-522, 1995); bovine indolicidin (Del Sal et al., *Biochem. Biophys. Res. Commun.* **187**:467-472, 1992); the porcine antimicrobial peptides prophenin-2 and PR-39 (Zhao et al., *FEBS Lett.* **367**:130-134, 1995) and PMAP-37 (Tossi et al., *Eur. J. Biochem*, **15**:941-946, 1995); the human antimicrobial lipopolysaccharide binding protein CAP18 (Larrick et al., *Infect. Immun.* **63**:1291-1297, 1995); and the murine protein E3 (Scott and Collins, *Blood* **88**:2517-2530, 1996).

While the anionic pro-region peptide may be directly joined to the N-terminus of the cationic peptide, an alternative embodiment involves linking the pro-region peptide to the dermaseptin or temporin peptide using a spacer peptide sequence. The use of spacer peptides to join two peptide domains is well known in the art; such spacer peptides are typically of between 2 and 25 amino acids in length, and provide a flexible hinge connecting the first peptide sequence to the second peptide. Spacer sequences that have been used to provide flexible hinges connecting two peptide sequences include the glycine(4)-serine spacer (GGGGS x3) described by Chaudhary et al., *Nature* **339:** 394-397, 1989. Alternatively, an N-terminal peptide extension as described below may serve to provide the spacer peptide function. Spacer sequence peptides may also include a cleavage site, such as a peptide sequence recognized and cleaved by a protease, such as Factor Xa. Such sites facilitate removal of the pro-region from the dermaseptin or temporin peptide following purification from plant tissues. The use of anionic pro-region peptides and spacer peptides to express certain cationic peptides in microbial systems is known in the art and described in US Patent 5,593,866 to Hancock.

In certain embodiments, an N-terminal extension peptide sequence may be added to the dermaseptin or temporin peptide. Such peptide extensions may comprise portions of the precursor forms of dermaseptins or temporins that are usually removed during protein processing, or may be synthetic sequences. These N-terminal peptide extensions may serve to provide enhanced resistance to proteolytic cleavage, enhance transcription levels, or enhance the antimicrobial activity of the peptides. Typically, these N-terminal extensions are of between 2 and 25 amino acids in length, although longer extensions may also be employed. Examples of N-terminal extension sequences that are utilized in certain embodiments include the peptide sequences AMWK, ASRH, and ALWK. The AMWK (SEQ ID: 39) sequence is a naturally-occurring peptide extension; it is part of the full-length dermaseptin-b peptide sequence that is normally cleaved during processing. The addition of this sequence to the N-terminus of dermaseptin b (to produce dermaseptin B) has been reported to enhance the in vitro antimicrobial activity of the peptide (Strahilevitz, *Biochemistry*, **33**:10951-10960, 1995). The ASRH (SEG ID: 41), and ALWK (SEQ ID:41) peptides are synthetic extension sequence. In each case, an N-terminal methionine is added to ensure proper expression of the peptide. One of skill in the art will appreciate that the effect of adding

any particular N-terminal extension peptide on the biological activity of the peptide being produced (dermaseptin or temporin) may readily be assessed using the biological activity assay described above.

d. Variant Dermaseptin and Temporin Peptides

As described above, a number of naturally occurring temporin and dermaseptin peptides are known, exemplified by those shown in the Sequence Listing. Variants on these naturally occurring peptides may be selected by introducing amino acid substitutions, additional amino acid residues, or by deleting amino acid residues. These variant peptides may either be produced by chemical synthesis (for example, in order to confirm that the variant peptide retains functional activity), or may be produced in a biological expression system. In the latter instance, the nucleic acid sequence encoding the corresponding naturally occurring peptide can be manipulated so that it encodes the variant peptide. This can be done through a variety of methods, for example by using site-directed mutagenesis or the polymerase chain reaction. Alternatively, because the peptides are relatively short molecules, the coding region for a variant peptide can simply be synthesized *de novo* and introduced into a suitable expression vector.

The simplest modifications of amino acid sequences involve the substitution of one or more amino acids for amino acids having similar biochemical properties. These so-called conservative substitutions are likely to have minimal impact on the activity of the resultant protein. Thus, peptides that differ from naturally occurring temporin or dermaseptin peptides by one or more conservative amino acid substitutions may be used in the invention in place of the naturally occurring peptides. Table 1 shows amino acids which may be substituted for an original amino acid in a protein and which are regarded as conservative substitutions.

Table 1

Original Residue	Conservative Substitutions
Ala	ser
Arg	lys
Asn	gln; his
Asp	glu
Gln	asn
Glu	asp
Gly	pro
His	asn; gln
Ile	leu; val
Leu	ile; val
Lys	arg; gln; glu
Met	leu; ile
Phe	met; leu; tyr
Ser	thr
Thr	ser
Trp	tyr
Tyr	trp; phe
Val	ile; leu

More substantial changes in functional or other features may be obtained by selecting substitutions that are less conservative than those in Table 1, i.e., selecting residues that differ more significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. The substitutions which in general are expected to produce the greatest changes in protein properties will be those in which (a) a hydrophilic residue, e.g., seryl or threonyl, is substituted for (or by) a hydrophobic residue, e.g., leucyl, isoleucyl, phenylalanyl, valyl or alanyl; (b) a cysteine or proline is substituted for (or by) any other residue; (c) a residue having an electropositive side chain, e.g., lysyl, arginyl, or histidyl, is substituted for (or by) an electronegative residue, e.g., glutamyl or aspartyl; or (d) a residue having a bulky side chain, e.g., phenylalanine, is substituted for (or by) one not having a side chain, e.g., glycine. Variant peptides having one or more of these more substantial changes may also be employed in the invention, provided that temporin or dermaseptin biological activity is retained.

More extensive amino acid changes may also be engineered into variant dermaseptin or temporin peptides. As noted above however, these variant peptides will typically be characterized by possession of at least 40% sequence identity counted over the full-length alignment with the amino acid sequence of their respective naturally occurring sequences using the alignment programs described above. In addition, these variant peptides will retain biological activity.

Confirmation that a dermaseptin or temporin peptide has biological activity may be achieved using the assay systems described above. Following confirmation that the peptide has the desired activity, a nucleic acid molecule encoding the peptide may be readily produced using standard molecular biology techniques. Where appropriate, the selection of the open reading frame will take into account codon usage bias of the plant species in which the peptide is to be expressed.

III. Introducing Dermaseptins and/or Temporins into Plants

Once a nucleic acid sequence encoding a dermaseptin and/or a temporin peptide has been produced, standard techniques may be used to express the sequence in transgenic plants in order to confer pathogen resistance to the plant. The basic approach is to clone the nucleic acid into a transformation vector, such that it is operably linked to control sequences (e.g., a promoter) that direct expression of the nucleic acid in plant cells. The transformation vector is then introduced into plant cells by one of a number of techniques (e.g., electroporation), whole plants are regenerated from the cells, and progeny plants containing the introduced nucleic acid are selected. Preferably all or part of the transformation vector will stably integrate into the genome of the plant cell. That part of the transformation vector which integrates into the plant cell and which contains the introduced sequence and associated sequences for controlling expression (the introduced "transgene") may be referred to as the recombinant expression cassette.

Selection of progeny plants containing the introduced transgene may be made based upon the detection of an altered phenotype. Such a phenotype may result directly from the disease resistance conferred by the introduced sequence or may be manifested as enhanced resistance to a chemical agent (such as an antibiotic) as a

result of the inclusion of a dominant selectable marker gene incorporated into the transformation vector.

Successful examples of the modification of plant characteristics by transformation with cloned nucleic acid sequences are replete in the technical and scientific literature. Selected examples, which serve to illustrate the knowledge in this field of technology include:

U.S. Patent No. 5,571,706 ("Plant Virus Resistance Gene and Methods")

U.S. Patent No. 5,677,175 ("Plant Pathogen Induced Proteins")

U.S. Patent No. 5,510,471 ("Chimeric Gene for the Transformation of Plants")

U.S. Patent No. 5,750,386 ("Pathogen-Resistant Transgenic Plants")

U.S. Patent No. 5,597,945 ("Plants Genetically Enhanced for Disease Resistance")

U.S. Patent No. 5,589,615 ("Process for the Production of Transgenic Plants with Increased Nutritional Value Via the Expression of Modified 2S Storage Albumins")

U.S. Patent No. 5,750,871 ("Transformation and Foreign Gene Expression in Brassica Species")

U.S. Patent No. 5,268,526 ("Overexpression of Phytochrome in Transgenic Plants")

U.S. Patent No. 5,780,708 ("Fertile Transgenic Corn Plants")

U.S. Patent No. 5,538,880 ("Method For Preparing Fertile Transgenic Corn Plants")

U.S. Patent No. 5,773,269 ("Fertile Transgenic Oat Plants")

U.S. Patent No. 5,736,369 ("Method For Producing Transgenic Cereal Plants")

U.S. Patent No. 5,610,042 ("Methods For Stable Transformation of Wheat").

These examples include descriptions of transformation vector selection, transformation techniques and the construction of constructs designed to over-express the introduced transgene.

a. Plant Types

Diseases caused by many pathogens affect a wide variety of plant species. These plants can be monocots, dicots or gymnosperms. Thus, for example, dermaseptins and/or temporin peptides may be introduced into plant species including, but not limited to, maize, wheat, rice, barley, soybean, cotton, beans in general, rape/canola, alfalfa, flax, sunflower, safflower, brassica, cotton, tobacco, flax, peanut, clover, cowpea, grapes; vegetables such as lettuce, tomato, cucurbits, cassava, potato, carrot, radish, pea, lentils, cabbage, cauliflower, broccoli, Brussels sprouts,

peppers; tree fruits such as citrus, apples, pears, peaches, apricots, walnuts; fur trees such as Douglas fir and loblolly pine, and flowers such as carnations and roses.

b. Vector Construction and Choice of Promoters

A number of recombinant vectors suitable for stable transfection of plant cells or for the establishment of transgenic plants have been described including those described in Pouwels et al., *Cloning Vectors: A Laboratory Manual*, 1985, supp., 1987; Weissbach and Weissbach, *Methods for Plant Molecular Biology*, Academic *Press*, **5**:173-184, 1989; and Gelvin et al., *Plant Molecular Biology Manual*, Kluwer Academic Publishers, 1990. Typically, plant transformation vectors include one or more cloned sequences under the transcriptional control of 5' and 3' regulatory sequences and a dominant selectable marker. Such plant transformation vectors typically also contain a promoter regulatory region (e.g., a regulatory region controlling inducible or constitutive, environmentally-or developmentally-regulated, or cell- or tissue-specific expression), a transcription initiation start site, a ribosome binding site, an RNA processing signal, a transcription termination site, and/or a polyadenylation signal. Examples of constitutive plant promoters that may be useful for expressing a transgene include: the cauliflower mosaic virus (CaMV) 35S promoter, which confers constitutive, high-level expression in most plant tissues (see e.g., Odel et al., *Nature*, **313**:810, 1985; Dekeyser et al., *Plant Cell*, **2**:591, 1990; Terada and Shimamoto, *Mol. Gen. Genet.* **220**:389, 1990; and Benfey and Chua, *Science*, **250**:959-966, 1990) the nopaline synthase promoter (An et al., *Plant Physiol.* **88**:547, 1988); the octopine synthase promoter (Fromm et al., *Plant Cell*, **1**:977, 1989) and the 2x CaMV/35S promoter with a translational enhancer sequence (Kay et al., *Science*, **236**:1299-1302, 1987).

A variety of plant gene promoters that are regulated in response to environmental, hormonal, chemical, and/or developmental signals, also can be used for expression of a transgene in plant cells, including promoters regulated by: (a) heat (Callis et al., *Plant Physiol.*, **88**:965, 1988; Ainley et al., *Plant Mol. Biol.*, **22**:13-23, 1993; and Gilmartin et al., *The Plant Cell*, **4**:839-949, 1992) (b) light (e.g., the pea rbcS-3A promoter, Kuhlemeier et al.. *Plant Cell*, **1**:471, 1989, and the maize rbcS promoter, Schaffner & Sheen, *Plant Cell*, **3**:997, 1991); (c) hormones, such as abscisic acid (Marcotte et al., *Plant Cell*, **1**: 471, 1989); (d) wounding (e.g., the potato

PinII promoter (Keil et al., *Nucl. Acids. Res.* **14**: 5641-5650, 1986), the *Agrobacterium* mas promoter (Langridge et al.. *Bio/Technology* **10**:305-308, 1989), and the grapevine vst1 promoter (Weise et al.. *Plant Mol. Biol.*, **26**:667-677, 1994); and (e) chemicals such as methyl jasmonate or salicylic acid (see also Gatz et al.. *Plant Mol. Biol.* **48**:89-108, 1997).

Alternatively, tissue specific (root, leaf, flower, and seed for example) promoters (Carpenter et al., *The Plant Cell* **4**:557-571, 1992; Denis et al., *Plant Physiol.* **101**:1295-1304, 1993; Opperman et al., *Science* **263**:221-223, 1993; Stockhause et al., *The Plant Cell* **9**:479-489, 1997; Roshal et al., *The EMBO* J. **6**:1155, 1987; Schernthaner et al., *EMBO J.* 7:1249, 1988; Yamamoto et al., *Plant Cell* **3**:371-382, 1990; and Bustos et al., *Plant Cell* 1:839, 1989) can be fused to the coding sequence to obtain particular expression in respective organs.

Plant transformation vectors may also include RNA processing signals, for example, introns, which may be positioned upstream or downstream of the ORF sequence in the transgene. In addition, the expression vectors may also include additional regulatory sequences from the 3'-untranslated region of plant genes, e.g., a 3' terminator region to increase mRNA stability of the mRNA, such as the PI-II terminator region of potato or the octopine or nopaline synthase (NOS) 3' terminator regions.

Finally, as noted above, plant transformation vectors may also include dominant selectable marker genes to allow for the ready selection of transformants. Such genes include those encoding antibiotic resistance genes (e.g., resistance to hygromycin, kanamycin, bleomycin, G418, streptomycin or spectinomycin) and herbicide resistance genes (e.g., phosphinothricin acetyltransferase).

c. Transformation and Regeneration Techniques

Transformation and regeneration of both monocotyledonous and dicotyledonous plant cells is now routine, and the appropriate transformation technique will be determined by the practitioner. The choice of method will vary with the type of plant to be transformed; those skilled in the art will recognize the suitability of particular methods for given plant types. Suitable methods may include, but are not limited to: electroporation of plant protoplasts; liposome-mediated transformation; polyethylene glycol (PEG) mediated transformation; transformation

using viruses; micro-injection of plant cells; micro-projectile bombardment of plant cells; vacuum infiltration; and *Agrobacterium tumefaciens* (AT) mediated transformation. Typical procedures for transforming and regenerating plants are described in the patent documents listed at the beginning of this section.

d. Selection of Transformed Plants

Following transformation and regeneration of plants with the transformation vector, transformed plants are usually selected using a dominant selectable marker incorporated into the transformation vector. Typically, such a marker will confer antibiotic resistance on the seedlings of transformed plants, and selection of transformants can be accomplished by exposing the seedlings to appropriate concentrations of the antibiotic.

Selection can also be accomplished by exploiting the pathogen resistance that is conferred to the plant via the transgene. As described in the Examples below, such screening may be accomplished either after the transgenic plants have been regenerated, or (depending on the transformation method used) may be performed on green transgenic callus prior to plant regeneration.

IV. Plants Containing Coding Regions for Multiple Cationic Peptides

In some cases, the level of resistance that is conferred by a single copy of a transgene encoding a dermaseptin or a temporin peptide may be enhanced by introducing multiple copies of a single cationic peptide gene, or several genes encoding different cationic peptides.

Through the use of genetic engineering it is possible to introduce coding regions for multiple cationic peptides into a single vector. Typically (though not necessarily) such vectors comprise two or more dermaseptin and/or temporin open reading frames each operably linked to its own 5' and 3' regulatory sequences. When introduced into plants, such vectors can result in the expression of multiple varieties of cationic peptides.

The creation of a plant containing multiple transgenes can also be accomplished through the use of standard breeding techniques. A transgene encoding a first cationic peptide can be introduced into a first plant and a second transgene encoding a second cationic peptide can be introduced into a second plant. The

resulting transgenic plants can then be crossed to produce progeny that carry both transgenes.

V. Production and Isolation of Dermaseptins and Temporins

The compositions and methods described above may be used not only to produce plants having enhanced, broad spectrum pathogen resistance, but may also be used for the large scale production of dermaseptins and temporins for a wide range of other applications. For example, temporins and dermaseptins produced in large quantities in plants may be purified and used in medical applications.

The production of biologically active peptides in plants is now widely practiced, and bulk expression and purification methods are well known. Examples of constructs that facilitate the production of biologically active proteins in plants can be found in U.S. Patent 4,956,282 to Goodman et al. These constructs generally contain a promoter region and an additional nucleic acid sequence that encodes an amino acid sequence that is later utilized in the purification process. The amino acid sequence that is used to facilitate the isolation of the dermaseptin and/or temporin peptides can be subsequently cleaved and discarded.

EXAMPLES

1. Selection and Creation of Nucleic Acid Sequences Encoding the Dermaseptin and the Temporin Peptides

a. Dermaseptin Coding Sequence

A nucleic acid molecule was designed to encode the mature 27 amino acid form of dermaseptin b (SEQ ID: 3) with a 5 amino acid N-terminal extension sequence, MAMWK. This nucleic acid construct was designated $MSRA_2$ and was synthesized using four overlapping oligonucleotides in a single PCR reaction. The oligonucleotides used are shown in Table 2. The first two oligonucleotides (oligo #1 and oligo #2) contained the nucleic acid sequences encoding the N-terminal and the C-terminal portions of the peptide, respectively. These oligonucleotides were used in the PCR reaction at a 20 nM concentration. The second two oligonucleotides contained sequences recognized by various restriction enzymes. Specifically, oligo #3 contained restriction sites for *Xba*I, *Kpn*I and *Nco*I, and oligo #4 contained

restriction site for *Sst*I, *Pst*I and *Hind*III. These oligonucleotides were used at a concentration of 200 nM in the PCR reaction. Following amplification of the product, it was cloned using the built-in restriction sites into a conventional cloning vector. The nucleic acid sequence of the coding region of $MSRA_2$ is shown in SEQ ID: 27, and the encoded peptide is shown in SEQ ID: 28. Oligo #s 1-4 are shown in SEQ IDs: 29-32.

Table 2

Oligo # 1: 5' - ATG GCC ATG TGG AAA GAC GTT CTG AAA AAG
(SEQ ID: 29) ATC GGT **ACT GTC GCC CTC CAT GCA GGG** - 3'

Oligo # 2: 3' - **TGA CAG CGG GAG GTA CGT CCC** TTC CGG CGC
(SEQ ID: 30) GAA CCT CGT CAT CGG CTG TGG TAG AGC GTC ATT - 5'

Oligo # 3: 5' - TCT AGA GGT ACC ATG GCC ATG TGG AAA GAC G -3'
(SEQ ID: 31)

Oligo # 4: 3' – GGC TGT GGT AGA GCG TCA TTC TCG AGA CGT CTT
(SEQ ID: 32) CGA AC – 5'

The nucleotides in bold represent the regions of complementarity between oligo #1 and oligo #2. The underlined portion of oligo #1 is identical to underlined portion of oligo #3, thus allowing oligo #3 to bind to the PCR product from the initial elongation of oligos #1 and #2. Similarly, the underlined portions of oligo #4 are identical to the underlined portion of oligo #2. This allows oligo #4 to bind to the PCR product created by elongation of oligos #1 and #2.

b. Temporin Coding Sequence

A nucleic acid molecule was designed to encode the mature 13 amino acid form of temporin A (SEQ ID:33) with a 6 amino acid N-terminal extension sequence, MASRHM. This nucleic acid construct was designated $MSRA_3$ and was synthesized using four overlapping oligonucleotides in a single PCR reaction. The oligonucleotides used are shown in Table 3. The first two oligonucleotides (oligo #1 and oligo #2) contained the nucleic acid sequences encoding the prototype peptide. However, unlike the oligonucleotides used to encode the prototype dermaseptin

peptide, these oligonucleotides were fully complementary, thus eliminating the need for an initial elongation cycle prior to the binding of ligos #3 and #4. Oligos #1 and #2 were used in the PCR reaction at a 20 nM concentration. The second two oligonucleotides contained sequences recognized by various restriction enzymes. Specifically, oligo #3 contained restriction sites for *Xba*I, *Kpn*I and *Nde*I, and oligo #4 contained restriction site for *Sst*I, and *Pst*I. These oligonucleotides were used at a concentration of 200 nM in the PCR reaction.

Following amplification of the product, it was cloned using the built-in restriction sites into a conventional cloning vector. The nucleic acid sequence of the coding region of $MSRA_3$ is shown in SEQ ID:33, and the encoded peptide is shown in SEQ ID: 34. Oligo #s 1-4 are shown in SEQ IDs: 35-38.

Table 3

Oligo # 1: 5' – **ATG TTT CTG CCC CTA ATC GGG AGG GTT CTC TCG**
(SEQ ID: 35) **GGA ATC CTG TAA** – 3'

Oligo # 2: 3' – **TAC AAA GAC GGG GAT TAG CCC TCC CAA GAG**
(SEQ ID: 36) **AGC CCT TAG GAC ATT** – 5'

Oligo # 3: 5' – GGT ACC TCT AGA CAT ATG TTT CTG CCC CTA – 3'
(SEQ ID:37)
Oligo # 4: 3' – GAG AGC CCT TAG GAC ATT CTC GAG ACG TC – 5'
(SEQ ID: 38)

The nucleotides in bold represent the regions of complementarity between oligo #1 and oligo #2. As depicted in the diagram these sequences are fully complementary. The underlined portions oligo #2 are complementary to the underlined portions of oligo #3 and the underlined portions of oligo #1 are complementary to the underlined portions of oligo #4.

The resulting double stranded DNA was then cloned into one or more of the vectors described below.

2. Vect rs Containing Vari us Pr moter Sequences

The nucleic acid sequences encoding $MRSA_2$ and $MRSA_3$ were assembled into various plant transformation vectors, thereby placing them under the transcriptional control of a variety of different promoters.

Cloning $MRSA_2$ and $MRSA_3$ sequences into one such vector placed the respective sequences under the control of a promoter that contained two copies of the CaMV 35S promoter and an AMV RNA4 translation enhancing element (Kay et al., *Science* **236**:1299-1302, 1987). The clones that resulted from ligation into this vector were given the prefix pD as a designation. Therefore, $pDMSRA_2$ designates a vector which contains the $MSRA_2$ construct under the control of the double CaMV 35S promoter and the AMV RNA4 translational enhancer. Similarly, $pDMSRA_3$ designates a vector that contains the $MSRA_3$ construct under the control of the double CaMV 35S promoter with the AMV RNA4 translational enhancer.

Another vector was designed such that the $MSRA_2$ construct was under the control of a rebuilt "super promoter". This promoter contained the mas (mannopine synthase) promoter/activator region (Langridge et al., *Bio/Technology* **10**:305-308, 1989) preceded by a trimer of ocs (octopine synthase) upstream activating sequence (in inverted orientation). A more detailed description of this super promoter is provided in Ni et al., *The Plant J.*, 7: 661-676, 1995. This particular construct also contained a coding region for a 6 x His tag, positioned upstream of and operably linked to the $MSRA_2$ coding region. The 6 x His tag amino acid sequence is thus expressed as an N-terminal addition to the encoded dermaseptin/ MAMWK peptide. The vector encoding this peptide was designated $pRSHMSRA_2$.

3. Transformation of Potato and Tobacco

Potato cultivars, Russert Burbank and Desiree, as well as tobacco were used as representative plant species for transformation. The transformation of the plants was performed according to DeBlock, *Theoret. Appl. Genet.* **76**:767-774, 1988, with some modifications.

Briefly, transformations of tobacco and potato were carried out by isolating leaves (5 mm) and stems from 4-week-old shoots. These leaves and shoots were cut and further wounded by scratching with glass pipette tips. Wounded leaves and stems

were then floated upside down on 15 ml of S2 medium in a petri dish. This medium contained the components listed in Murashige and Skoog, *Physiol. Plant.* **15**:473-479, 1962, supplemented with 30 g/L sucrose 0.5 g/L MES, pH 5.5, and 20 g/L mannitol.

60 µl of LB medium containing *Agrobacterium* (transformed with the vector of interest) in the late log phase of growth was added to each petri dish, and the dishes were then incubated at low light intensity (500 lux) for 3 days in the presence of the *Agrobacterium.* The plant tissue was then washed with S2 medium containing 1 g/L carbenicillin, blotted dry on filter paper, and placed upside down on S4 medium. S4 medium contained the components listed in Murashige and Skoog, *Physiol. Plant.* **15**:473-479, 1962 minus sucrose and supplemented with 200 mg/L glutamine, 0.5 g/L MES, pH 5.7, 0.5 g/L PVP, 20 g/L mannitol, 20 g/L glucose, 40 mg/L adenine-SO_4, 0.5% agarose, 1 mg/L trans-zeatin, 0.1 mg/L NAA, 1 g/L carbenicillin and 50 µg/ml kanamycin, 10 mg/L AgNO3). The plant tissue samples were placed at room temperature (RT) at 3000 lux to allow for callus formation. After two weeks, many small calli formed at the wounded edges of the leaves and stems. The small calli were removed and transferred to fresh S6 medium (S4 without NAA). After 2-3 weeks, the calli were transferred to S8 medium (S6 supplemented with 0.1 mg/L GA_3) to allow for shoot formation.

After two weeks on S8 medium, the first shoots (0.5 cm) were transferred to S1 medium. This medium contained the components described in Gamborg et al., *Exp. Cell Res.* **50**:151-158, 1968, with the addition of 20 g/L sucrose, 150 mg/L CaCl2, 0.4% agarose, pH 5.8, 1 g/L carbenicillin and 50 µg/ml kanamycin. The S1 medium and the shoots were placed in Magenta jars to allow for root formation. After one week the shoots had rooted. In order to avoid selecting identical shoots, transfer of shoots from the same or closely linked calli was avoided.

The regenerated putative transgenic plants were transferred to MS medium containing 1 g/L carbenicillin and 50 µg/ml kanamycin for further analysis.

4. Screening of Calli for Disease Resistance

A simplified early detection method for disease resistance assays was developed. Control and transgenic calli were grown on S4 medium (MS media without sucrose and supplemented with 200 mg/L glutamine, 0.5 g/L MES, pH 5.7,

0.5 g/L PVP, 20 g/L mannitol, 20 g/L glucose, 40 mg/L adenine-SO4, 0.5% agarose, 1 mg/L trans-zeatin, 0.1 mg/L NAA, 1 g/L carbenicillin and 50 µg/ml kanamycin, 10 mg/L AgNO3). The samples were then placed at RT at 3000 lux to allow for callus formation. After two weeks, many small calli formed at wounded edges of the leaves and stems. The small calli were removed and transferred to fresh S6 medium (S4 without NAA). After 2-3 weeks, the calli were transferred to fresh medium and grown in the presence of phytopathogens, *Fusarium* or *Phytophthora*. At the end of the experiments, calli that survived and stayed bright green were scored. No fungal resistant calli were found in the control samples, and calli that were resistant to the fungal pathogen were found to be transformed.

5. Molecular Characterization of Transgenic Plants

DNA was isolated from the transgenic potato and tobacco plants using the methods described below. In some instances purification involved a more rigorous protocol and in others a simple crude extract procedure was performed. The more rigorous extract procedure started by obtaining ten grams of fresh leaf tissue, and immediately freezing the sample in liquid nitrogen. The frozen tissue was then ground into a fine power and extracted with 20 ml extraction buffer (50 mM Tris-HCl buffer, 5 mM EDTA, 0.35 M sorbitol, 0.1% BSA, 0.1% β mercaptoethanol, 10% PEG 4000). The homogenate was filtered through several layers of cheesecloth and one layer of miracloth. The final purification steps were then performed in accordance with Wagner et al., *Proc. Natl. Acad. Sci. U.S.A.* **84**:2097-5100, 1987.

The crude extract procedure was used mainly when isolation was being done for the purposes of PCR analysis. For this procedure about 200 mg of fresh leaves were collected and ground in liquid nitrogen into a powder. 100 µl of 0.5 N NaOH was added to the powder and mixed (vortexed) for 30 seconds. The suspension was centrifuged for 5 minutes and 5 µl of the supernatant was added into 45 µl of 100 mM of Tris buffer (pH 8.0). This crude genomic DNA extract was used as the template for PCR amplification.

Detection of the presence or absence f the $MSRA_2$ construct was achieved by performing a PCR reaction with the extracted genomic DNA and oligos # 3 and # 4 from Table 2 above. The expected size of the product from the reaction was 129 bp. This method allowed for the identification of transgenic tobacco and potato plants transformed with the $pDMSRA_2$ or $pRSHMSRA_2$ constructs.

Detection of the $MSRA_3$ construct was achieved by performing PCR reactions either with oligo #3 and oligo # 4 from Table 3 above or with a primer specific for the 2 x 35S CaMV promoter in combination oligo #4 from Table 3 above. Transgenic tobacco plants containing the $pDMSRA_3$ construct were identified.

A control plant was also engineered to contain a GUS gene under the control of a super promoter containing the mas (mannopine synthase) promoter/activator region preceded by a trimer of the ocs (octopine synthase) upstream activating sequence (Ni et al., *The Plant J.* 7: 661-676. 1995). The insertion of this transgene into the tobacco plant genome was confirmed via PCR techniques.

In some cases, active expression of the transgene was confirmed by Northern blot analysis. The RNA substrate for these experiments was isolated and purified from the transgenic tobacco and potato plants. The protocol used for this isolation was performed in accordance with Verwoerd et al., *Nucl. Acids Res.* 17:2362, 1989.

6. Resistance to Bacterial Pathogens

To examine the resistance of the transgenic potato and tobacco plants to the bacterial pathogen *Erwinia carotovora*, the bacteria were grown in LB media at RT overnight (A600= 2.9). A 2 ml aliquote of *Erwinia* culture was diluted 5 times in dH_2O and 1 ml of the diluted culture was added to 2 ml MS liquid media and used in the antibacterial assay. Freshly cut branches (~3.5 cm) from transgenic plants or control plants were inserted into the tubes containing the diluted *Erwinia* culture so that the bottom of the cut edge of the plant was immersed into the bacterial culture. The test tubes were incubated at RT at 3000 lux and observed intermittently.

Transgenic potato plants containing $pDMSRA_2$ and transgenic tobacco plants containing either $pDMSRA_2$ or $pRSHMSRA_2$ were tested as described and showed resistance to the pathogen: After one week of growth in the presence of the bacterial culture the transgenic plants were uninfected (as determined by visual inspection)

and continued to grow. In sharp contrast, a control plant challenged with bacterial culture was severely infected after one week of incubation, growth was inhibited and the plant died after 2-3 weeks of exposure to fungal pathogens.

To examine the resistance of transgenic potato tubers to the bacterial pathogen *Erwinia carotovora* cv *carotovora*, a small well was made into tuber discs (2 cm diameter, 3 cm thick). Twenty microliters of 100x diluted overnight bacterial culture (approximately $2x10^7$ CFU) was pipetted into the well and discs were incubated at room temperature for 6 days. Rotted tissue was then gently removed from the tuber discs and the loss of weight of the tissue determined. Results obtained from such assays showed that compared to the non-transgenic control the transgenic potato tissue is resistant to soft rot (Figure 1).

Bactericidal effects of antimicrobial peptides (SEQ ID NO: 34 ($MRSA_3$) and SEQ ID NO: 28 ($MRSA_2$)) were determined against *Escherichia coli* (Figure 2) and *Erwinia carotovora* (Figure 3) in microtiter plates in a final volume of 220 µl containing approximately $1x10^5$ bacteria/ml and indicated concentration of antimicrobial peptides. The cell cultures were incubated at room temperature for 4 hours, diluted and plated on LB plates. After overnight incubation at 37°C (*E. coli*) or 28°C (*E. carotovora*) the colonies were counted and the survival of bacteria was scored. Results from both assays showed that the peptides have significant antimicrobial activity.

7. **Resistance to Fungi**

Mature plants were tested for their resistance to various fungi using the following protocol. One cm^2 x 0.5 cm of *Fusarium* or *Phytophthora sp.* -containing media slices were cut and put in the center of fresh plates of V8 agar media (250 ml/L V8 juice, 7 grams/L agar) in a 9 cm petri dish and grown for about one month at room temperature, or until the fungal mycelia completely covered the petri dish. Shoots of transgenic plants (~10 cm) were cut and transferred into MS medium for further growth. According to different treatments, plants were allowed to grow for 3 days or 2 weeks until the shoots rooted. Two 1 cm^2 x 0.5 cm slices of the fungal agar were then applied to both sides of the plant shoots without wounding the plant. The resulting degree of infection was then determined visually.

In a representative experiment, a transgenic potato plant transformed with pDMSRA$_2$, tobacco plants transformed with either pRSHMRSA$_2$ or pDMSRA$_2$, and control potato and tobacco plants were exposed to *Phytophthora cactorum*. After 7 days, *Phytophthora cactorum* had grown over the surface of MS medium, and penetrated into the roots and the stems of the control plants, causing impairment of vital plant functions. It was apparent that the roots in the control plants were severely damaged. The interaction between plants and fungi caused the secretion of yellow-brown pigments indicative of the decay process. Subsequently, the plants lost water and leaves became curly, the bottom of the stems softened, and the roots died. However, the transgenic plants stayed healthy and had no disease symptoms even though the fungal mycelia completely covered the MS media.

The experiment described above was also used to test the transgenic plant's resistance to *Phytophthora infestans*. The results from these assays showed that the transgenic plants were also resistant to *Phytophthora infestans*.

Another experiment was performed challenging a pDMSRA$_2$ transgenic potato plant with *Fusarium solani*. After 6 days, *Fusarium* grew all over the surface of MS medium, the damage to the roots in the control plants was severe, and the base of stem was penetrated by *Fusarium* and the stems were softened and veins of infected leaves showed clear browning and necrosis. After several days, the control plant collapsed and died. However, the transgenic plant continued to grow even under the extreme fungal infestation by *Fusarium solani*.

Having illustrated and described the principles of the invention in multiple embodiments and examples, it should be apparent to those skilled in the art that the invention can be modified in arrangement and detail without departing from such principles. We claim all modifications coming within the spirit and scope of the following claims.

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Claims

1. A transgenic plant expressing a cationic peptide selected from the group consisting of:

(a) temporins; and

(b) dermaseptins.

2. A transgenic plant comprising a recombinant nucleic acid molecule, wherein the nucleic acid molecule encodes a peptide selected from the group consisting of:

(a) temporins; and

(b) dermaseptins.

3. A transgenic plant according to claim 2 wherein peptide comprises an amino acid sequence selected from the group consisting of the amino acid sequences set forth in SEQ IDs: 3-14, and 17-26.

4. A transgenic plant according to claim 3 wherein the peptide further comprises an N terminal peptide extension of between 2 and 25 amino acids in length.

5. A transgenic plant according to claim 4 wherein the N terminal peptide extension is selected from the group consisting of AMWK, ASRH, and ALWK.

6. A transgenic plant comprising a recombinant nucleic acid molecule, wherein the nucleic acid molecule encodes a fusion peptide having a formula selected from the group consisting of:

(a) P-D ; and

(b) P-T,

wherein D is a dermaseptin peptide, T is a temporin peptide and P is an anionic pro-region peptide.

7. A transgenic plant comprising a recombinant nucleic acid molecule, wherein the nucleic acid molecule encodes a fusion peptide having a formula selected from the group consisting of:

(a) P-S-D ; and

(b) P-S-T,

wherein D is a dermaseptin peptide, T is a temporin peptide, P is an anionic pro-region peptide and S is a spacer peptide.

8. A transgenic plant comprising a nucleic acid molecule encoding a peptide comprising an amino acid sequence selected from the group consisting of:

(a) SEQ IDs: 3-14 and fragments thereof;

(b) amino acid sequences that differ from an amino acid sequence specified in (a) by one or more conservative amino acid substitutions; and

(c) amino acid sequences that share at least 40% sequence identity with an amino acid sequence specified in (a),

wherein the peptide has dermaseptin biological activity.

9. A transgenic plant according to claim 8 wherein the peptide further comprises an anionic pro-region peptide operably linked to the N-terminus of the peptide.

10. A transgenic plant comprising a nucleic acid molecule encoding a peptide comprising an amino acid sequence selected from the group consisting of:

(a) SEQ IDs: 17-26 and fragments thereof;

(b) amino acid sequences that differ from an amino acid sequence specified in (a) by one or more conservative amino acid substitutions; and

(c) amino acid sequences that share at least 50% sequence identity with an amino acid sequence specified in (a),

wherein the peptide has temporin biological activity.

11. A transgenic plant according to claim 8 wherein the peptide further comprises an anionic pro-region peptide operably linked to the N-terminus of the peptide.

12. A transgenic plant comprising a recombinant nucleic acid molecule encoding a peptide comprising an amino acid sequence selected from the group consisting of SEQ IDs: 28 and 34.

13. A method of producing a biologically active cationic peptide comprising:

providing a transgenic plant according to claim 1; and

isolating at least one biologically active cationic peptide from the plant.

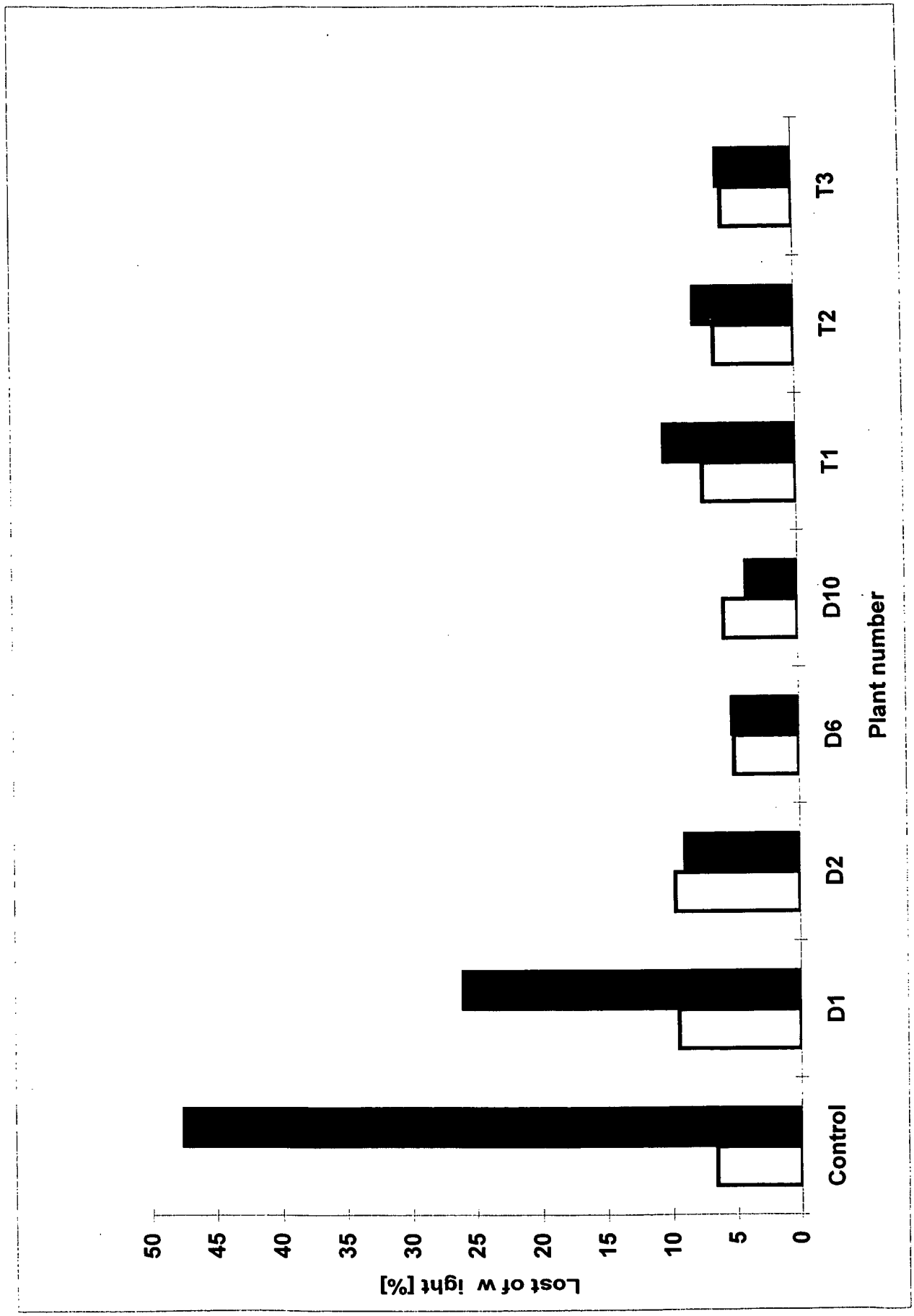
Lost of w ight [%]
0
5
10
15
20
25
30
35
40
45
50
Control
D1
D2
D6
D10
T1
T2
T3
Plant number

Figure 1

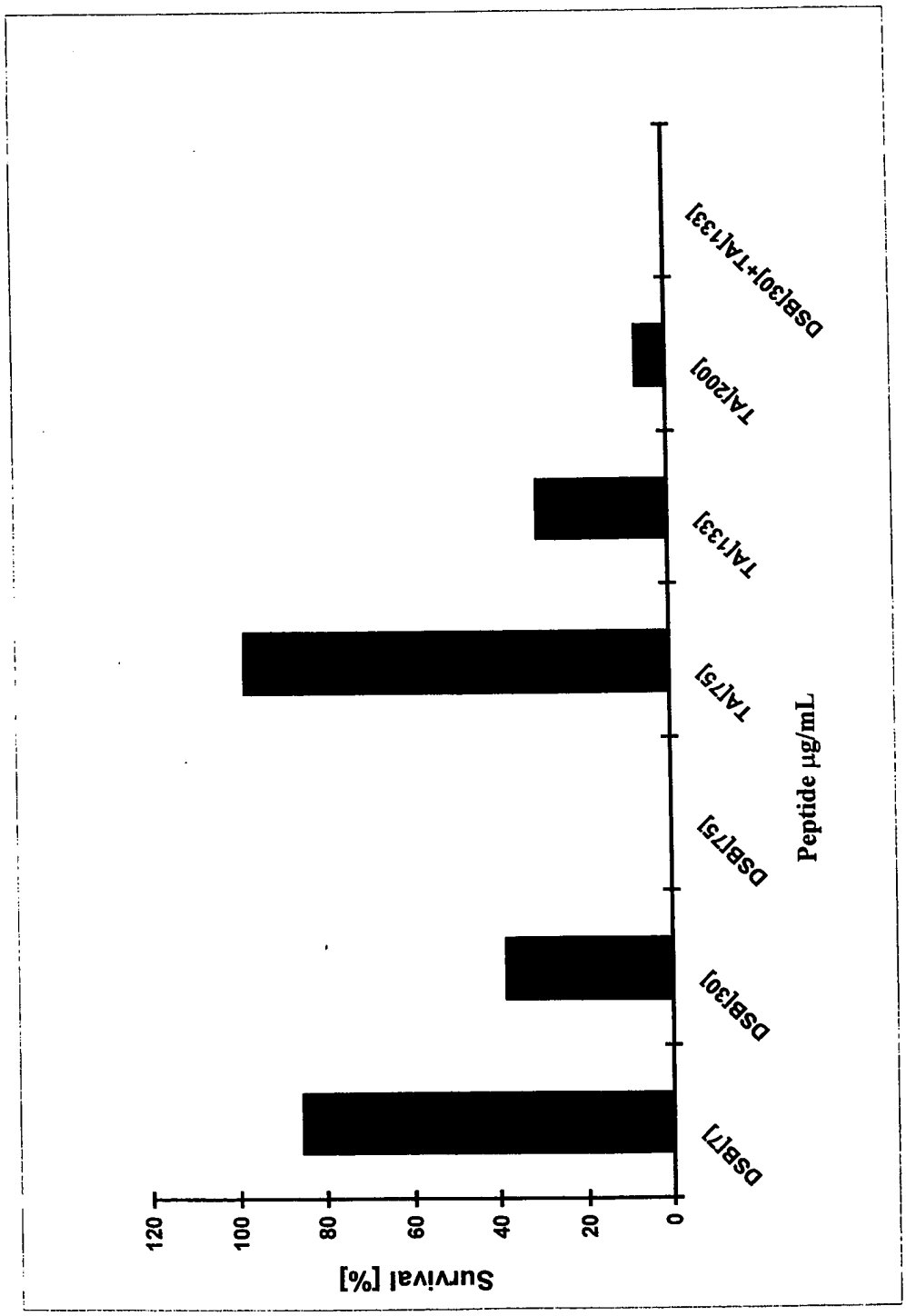
Survival [%]
120
100
80
60
40
20
0
DSBl7l
DSBl30l
DSBT5l
TAT5l
TAl133l
TAl200l
DSBl30l+TAl133l
Peptide µg/mL

Figure 2

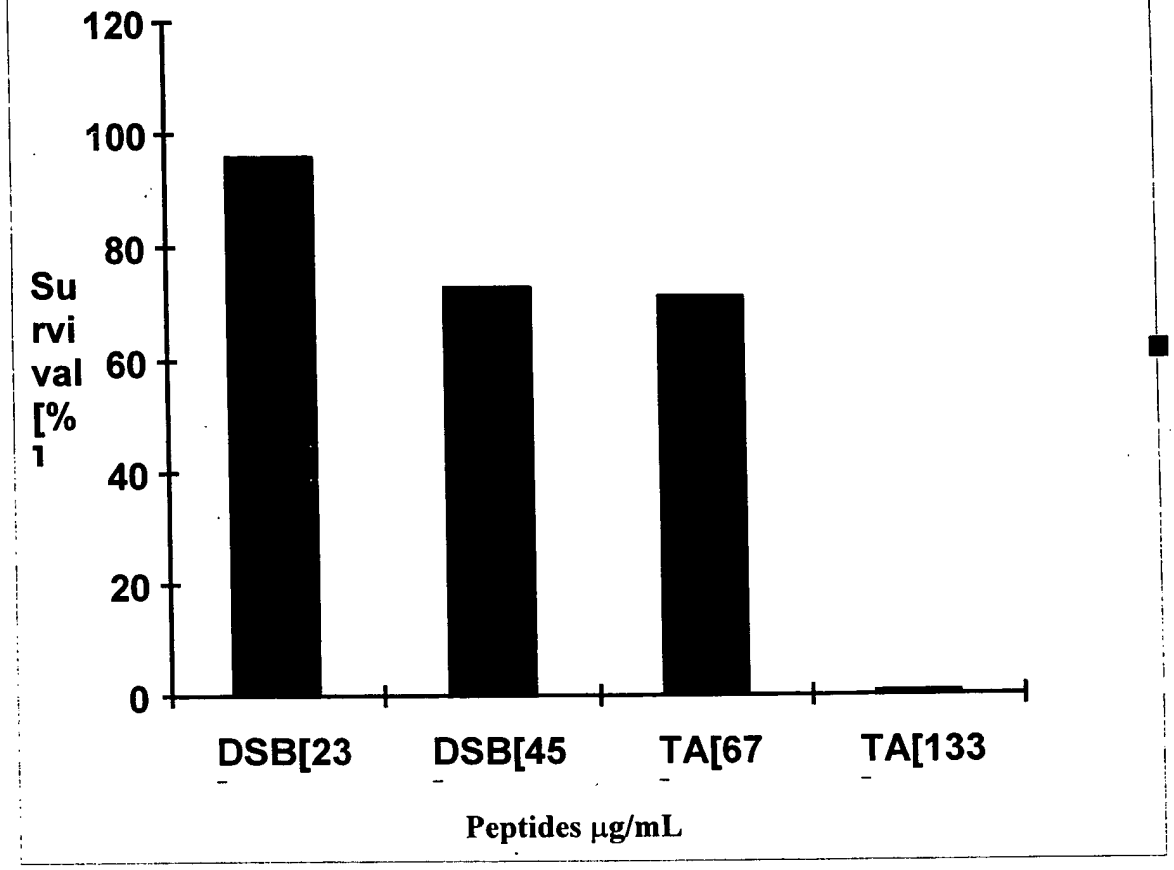
120
100
80
60
40
20
0
Survival [%]
DSB[23
DSB[45
TA[67
TA[133
Peptides μg/mL

Figure 3

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
3055-20/PAR		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/CA 00/ 00288	16/03/2000	17/03/1999

Applicant

University Of Victoria Innovation and Dev.....

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 5 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [X] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [X] furnished subsequently to this Authority in computer readble form.

   [X] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [X] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [X] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA 00/00288

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/82 C12N15/12 C12N15/62 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS, WPI Data, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 99 06564 A (ECK JOYCE VAN ;BLOWERS ALAN D (US); SANFORD JOHN (US); SMITH FRANZ) 11 February 1999 (1999-02-11) the whole document ---	1-5,8, 10,12,13
Y	SIMMACO M ET AL: "TEMPORINS, ANTIMICROBIAL PEPTIDES FROM THE EUROPEAN RED FROG RANA TEMPORARIA" EUROPEAN JOURNAL OF BIOCHEMISTRY,DE,BERLIN, vol. 242, no. 242, December 1996 (1996-12), pages 788-792-792, XP000856339 ISSN: 0014-2956 the whole document --- -/--	1-5,8, 10,12,13

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 July 2000	28/07/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Maddox, A

3

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; 14 October 1998 (1998-10-14) WECHSELBERGER CHRISTIAN: "Cloning of cDNAs encoding new peptides of the dermaseptin-family." Database accession no. PREV199800505321 XP002142520 abstract & BIOCHIMICA ET BIOPHYSICA ACTA, vol. 1388, no. 1, 14 October 1998 (1998-10-14), pages 279-283, ISSN: 0006-3002	1-5,8, 10,12,13
Y	MOR AMRAM ET AL: "The NH-2-terminal alpha-helical domain 1-18 of dermaseptin is responsible for antimicrobial activity." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, no. 3, 1994, pages 1934-1939, XP002142516 ISSN: 0021-9258 the whole document	1-5,8, 10,12,13
Y	STRAHILEVITZ JACOB ET AL: "Spectrum of antimicrobial activity and assembly of dermaseptin-b and its precursor form in phospholipid membranes." BIOCHEMISTRY, vol. 33, no. 36, 1994, pages 10951-10960, XP002142517 ISSN: 0006-2960 the whole document	1-5,8, 10,12,13
A	CHARPENTIER STEPHANE ET AL: "Structure, synthesis, and molecular cloning of dermaseptins B, a family of skin peptide antibiotics." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 273, no. 24, 12 June 1998 (1998-06-12), pages 14690-14697, XP002142518 ISSN: 0021-9258 the whole document	1-13

-/--

3

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MOR AMRAM ET AL: "The Vertebrate Peptide Antibiotics Dermaseptins Have Overlapping Structural Features but Target Specific Microorganisms." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, no. 50, 1994, pages 31635-31641, XP002142519 ISSN: 0021-9258 the whole document	1-13
A	WO 98 25961 A (BARRA DONNATELLA ;SIMMACO MAURIZIO (IT); SBL VACCIN AB (SE)) 18 June 1998 (1998-06-18) the whole document	1-13
A	EP 0 552 559 A (UNILEVER PLC ;UNILEVER NV (NL)) 28 July 1993 (1993-07-28) the whole document	1-13
A	EP 0 497 366 A (DONEGANI GUIDO IST) 5 August 1992 (1992-08-05) the whole document	1-13
A	EP 0 798 381 A (NAT INST AGROBIO RES) 1 October 1997 (1997-10-01) the whole document	1-13
A	WO 95 18855 A (PIONEER HI BRED INT) 13 July 1995 (1995-07-13) the whole document	1-13
A	WO 96 28559 A (UNIV BRITISH COLUMBIA) 19 September 1996 (1996-09-19) the whole document	6,7,9,11
A	WO 98 40401 A (FRASER JANET R ;MCNICOL PATRICIA J (CA); MICROLOGIX BIOTECH INC (C) 17 September 1998 (1998-09-17) the whole document	1-13
A	WO 98 06860 A (CIBA GEIGY AG ;VERNOOIJ BARNARDUS THEODORUS M (NL); CHANDLER DANIE) 19 February 1998 (1998-02-19) page 47	13
A	WO 98 50543 A (LEGRAND DOMINIQUE ;BIOCEM S A (FR); MEROT BERTRAND (FR); SALMON VA) 12 November 1998 (1998-11-12) the whole document	13
E	WO 00 31279 A (BARTFELD DANIEL ;BURIAN JAN (CA); MICROLOGIX BIOTECH INC (CA)) 2 June 2000 (2000-06-02) page 5, line 8 - line 19 page 15; claim 17	1,2,13

3 -/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
E	WO 00 26344 A (INTERLINK BIOTECHNOLOGIES LLC ;UNIV KENTUCKY RES FOUND (US)) 11 May 2000 (2000-05-11) claim 10	1,2,13

3

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/CA 00/00288

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9906564 A	11-02-1999	AU 8682098 A	22-02-1999
		EP 0990033 A	05-04-2000
WO 9825961 A	18-06-1998	AU 5501498 A	03-07-1998
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		CA 2060455 A	02-08-1992
		EP 0919566 A	02-06-1999
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		US 5519115 A	21-05-1996
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		AU 1557795 A	01-08-1995
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		EP 0738320 A	23-10-1996
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		EP 0815247 A	07-01-1998
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		EP 0918873 A	02-06-1999
		PL 331563 A	19-07-1999
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		AU 7659298 A	27-11-1998
		EP 0981617 A	01-03-2000
WO 0031279 A	02-06-2000	NONE	
WO 0026344 A	11-05-2000	NONE	

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Writer's Ext. **283**

Serial No.: PCT/CA00/00288

Applicant: University of Victoria Innovation and Development Corporation et al.

Title: **TRANSGENIC PLANTS THAT ARE RESISTANT TO A BROAD SPECTRUM OF PATHOGENS**

International Filing Date: 16/03/2000

Examiner: Loubradou-Bourges, N

Date: March 16, 2001

DELIVERED VIA FACSIMILE (Confirmation by Regular mail)
(Fax No. 011-49-89-2399-4465)

REPLY and ARTICLE 34 AMENDMENT

International Preliminary Examining Authority
European Patent Office
Erhardstrasse 27
D-80298 München
Germany

Dear Sirs:

This Reply and Amendment is responsive to the Written Opinion dated December 19, 2000, in the above-identified International Application.

Amendments

Enclosed is substitute page 6 and substitute page 40.

Substitute page 6 is identical to the originally filed page 6, except that a description of the black and white boxes has been added to the description of Figure 1. Support for this amendment can be found on pages 32-33 of the specification.

Substitute page 40 is identical to the original page 40 except that claim 14 has been added. Support for this new claim can be found on page 26, lines 6-19 of the specification.

Claims 1-13 were pending in the application and are unchanged.

A new claim 14 is presented for consideration.

Objections Stated in the Written Opinion

The Written Opinion contains objections relating to novelty, inventive step, and clarity.

The objections are addressed below.

Novelty and Inventive Step

Claims 1, 2 and 13 were rejected as allegedly lacking novelty in view of WO 99/06564 (Sanford Scientific Inc.).

Claims 1-13 were rejected as allegedly lacking an inventive step in view of WO 99/06564; WO 95/18855; and EP 0 552 559.

It is respectfully submitted that these objections are in error and should be withdrawn.

Claims 1-11 and 13-14

Claims 1-11 of the present application are directed to transgenic plants expressing a temporin and/or dermaseptin cationic peptide.

In paragraph 1 of Section V of the Written Opinion, it is asserted that:

> "the definition of temporins and dermaseptins includes structurally undefined variants ... [and] the only limitation is their antimicrobial activity. . . Thus, the Magainin antimicrobial cationic peptide may be considered to represent a modified dermaseptin or temporin."

Claims 13-14 are directed to a method for producing and isolating a dermaseptin or temporin peptide that is biologically active.

Paragraph 1 of Section V of the Written Opinion asserts that the suggestion in WO 99/06564 that magainin peptides can be purified from a transgenic plant, negates the novelty of claim 13.

Applicant respectfully disagrees with these conclusions stated in the Written Opinion.

Those skilled in the art of cationic peptides understand that there is a broad class of antimicrobial cationic peptides, which is divided into several distinct families, including magainins, temporins, and dermaseptins.

The magainin peptide disclosed in WO 99/06564 is in a separate family from the dermaseptin family and the temporin family.

Within each distinct family, there are homologues that have variant sequences. But all those homologues have the characteristics that are unique to their family. For example, as discussed on page 7, lines 4-23 of the present application, members of the dermaseptin family include, but are not limited to: dermaseptin S, dermaseptin–b, dermaseptins 1-5, as well as other dermaseptin-containing clones. Members of the temporin family include, but are not limited to: temporins A, B, C, D, E, F, G, H, K, and L (see specification page 10, lines 15-29).

(In addition to naturally occurring variant family members, alternative variant peptides can be generated using methods known to those skilled in the art. Each of those alternative variant peptides will be a member of the dermaseptin or temporin family, if it retains the unique characteristics and biological activity of the dermaseptin

or temporin family, as discussed on pages 8-12 of the specification.)

The magainin peptide disclosed in WO 99/06564 does not represent a modified dermaseptin or temporin, because the magainin peptide does not have the unique characteristics of the peptides of peptides of the temporin family or the dermaseptin family.

One problem in the art of expressing antimicrobial cationic peptides in plants is the potential for "phytotoxic effects exerted by the antimicrobial peptides when expressed in plants..." (page 1, lines 23-24 of WO 99/06564).

Previously it was not known whether dermaseptins or temporins could be expressed at levels necessary to allow isolation of the peptide from the plant, without causing toxic effects on the transgenic plant. As disclosed in the present application, dermaseptins and temporins, including variants thereof (see Figures 1-3) can be expressed in plants at levels sufficient to confer antimicrobial activity and for isolation of the peptide from the transgenic plant. But again, this was unknown prior to the work described in the present application.

In paragraph 2.1 of Section V of the Written Opinion, it is asserted that "the use of cationic peptides for [generating transgenic plants resistant to phytopathogens] is known ... and that the contribution of the present application is the provision of additional or further phytopathogen resistant plants...[and that] because the use of antimicrobial cationic peptides in general and of magainins in particular ...it is not sufficient to merely suggest further subgroups of cationic peptides known to have antimicrobial effects."

Applicant respectfully disagrees.

Although WO 99/06564 may disclose magainin or PGL "transgenic plants," there is no demonstration that the transgene transformed into the plants was actually incorporated into the plant genome or that the transgene was expressed. This is further evidenced by the fact that many "transgenic plants" did not tolerate the pathogens any better than the non-transformed plants (for example see WO 99/06564 Table 8, clones 320-6 and 169-33; Table 9, all clones except 319-7, Table

12, all except clone 168-7). Although WO 99/06564 demonstrates antimicrobial activities in the crude plant extracts, it is well known to those skilled in the art that the expression of natural host antimicrobial activities (acquired resistance) can occur nonspecifically in transgenic plants. In contrast, the present inventors demonstrate that dermaseptin and temporin (including variants) are transcribed, and have recently shown that biologically active peptides can be repurified from the plant, demonstrating the peptide is translated.

Even if the transgenic plants disclosed in WO 99/06564 express magainin or PGL, there is *no teaching or suggestion that expression of other cationic peptides such as dermaseptins and temporins would provide antimicrobial activity when expressed in a plant.* Instead, unpredictability is reported (WO 99/06564; pages 1-3). First, expression of antimicrobial peptides in plants has led to *inconsistent and often times unsatisfactory results* (WO 99/06564 page 1, lines 19-20; page 3, lines 4-9). This is supported by the statement that it was uncertain whether expression of magainin or PGL would confer resistance to the desired pathogens (WO 99/06564, page 3, lines 26-28). Second, there is concern regarding "*potentially phytotoxic effects* exerted by the antimicrobial peptides when expressed in plants..." (page 1, lines 23-24 of WO 99/06564). Third, the *amount of peptide expressed may need to reach a threshold level,* which may not be easily obtained, to obtain desired level of disease resistance (WO 99/06564, page 2, lines 27-28). Therefore, until the present work, it was unknown (1) whether a plant could tolerate the expression of dermaseptins or temporins (i.e. whether these peptides would be toxic to the plant), (2) whether levels of such expression could be achieved to obtain a desired level of disease resistance, and (3) whether such expression would give rise to disease resistance in the plant.

In paragraph 2.2 of Section V, it is asserted that "the disclosure of transgenic plants expressing [the particular SEQ ID NOs] does not involve an inventive activity."

Applicant respectfully disagrees.

The present inventors demonstrate a *new and unexpected effect* in that expression of demaseptins and temporins, including variants thereof (see Figur s 1-3), is well-tolerated in a variety of plants at levels that achieve antimicrobial activity.

Also in paragraph 2.2 of Section V it is asserted that "the addition of an N terminal peptide ... is a matter of normal design procedure."

Applicant respectfully disagrees.

The present inventors were the first to use N-terminal extensions of dermaseptins and temporins to provide a balance between phytopathogenicity and reduced toxicity of the host plant (see page 3 lines 17-29 and page 26, line 21-page 27, line 32 of the present application).

Therefore, the subject matter of claims 1-11 is inventive.

In paragraph 2.3 of Section V, it is asserted that "the subject matter of claim 13 is not inventive."

Applicant respectfully disagrees.

The inventors are the first to demonstrate first time that dermaseptins and temporins, including variants, can be expressed in plants at levels to achieve antimicrobial activity, and at levels that allow the peptides to be isolated in a biologically active form.

Therefore the subject matter of claim 13, as well as that of claim 14, is inventive.

Claim 12

In paragraph 2.4 of Section V, it is asserted that the subject matter of claim 12 is not inventive on the grounds that there is "no comparative data showing a new or unexpected effect of the transgenic plants as claim in claim 12 in comparison with transgenic potato resistant to soft rot due to the expression of magainin as described in [EP 0552 559]."

Applicant respectfully disagrees.

EP 0552 559 merely demonstrates some bacterial resistance in a handful of magainin transgenic potato plants wounded at their foliar components. In contrast, all of the dermaseptin and temporin peptides, including variants thereof, tested by the present inventors significantly reduced soft rot (Figure 1). In addition the peptides were produced in active form in the tubers, which is the location that profound resistance to decay by *Erwinia* sp. occurs, not in the foliar part of the plant. Recent experiments by the inventors demonstrate that these transgenic tubers can stably store for >2 years, which is 4-5 times greater than control, non-transgenic tubers.

Section VIII

The Written Opinion contains several objections regarding the clarity of the application.

It is respectfully submitted that these objections are in error and should be withdrawn.

1.1

As discussed herein, the terms " dermaseptins" and "temporins" are <u>not</u> open definitions. Dermaceptins and temporins are distinct families recognized by those skilled in the art (Tossi *et al.*, 2000, *Biopolym.* 55:4-30; Simmaco *et al.*, *Eur. J. Biochem.* 242: 788-92).

It is asserted that the term "biological activity" is merely antimicrobial activity, and is thus not a distinguishable feature. Applicant respectfully disagrees. Biological activity encompasses both specificity (i.e. the range of microorganisms killed) and potency (i.e. efficacy of killing/µg), as well as other activities and characteristics unique to a family. For example, one feature of dermaseptin biological activity is its ability to inhibit bacterial growth by at least 10% at 7µg/ml (page 9, lines 17-20), while for temporin biological activity the concentration is 100 µg/ml (page 11, line 30-page 12, line 2).

1.2

The expression "biologically active cationic peptide" is clearly defined in the present specification on pages 9-12. Therefore, the expression is not open to interpretation.

1.3

The expressions "anionic pro-region peptide" and "spacer peptide" are terms used in the art. In addition, descriptions of these terms can be found in the present specification on pages 17, line 14-page 18, line 21. Therefore, the expressions "anionic pro-region peptide" and "spacer peptide" are not vague and unclear.

1.4

The expression "fragment" of an amino acid sequence is described in the present specification on page 16, lines 1-3 and lines 28-31. A "fragment" is any portion of a full-length peptide that retains dermaceptin or temporin biological activity. Therefore, one skilled in the art would not interpret the term "fragment," as used in the present specification, to refer to a single amino acid, since a single amino acid will not have dermaceptin or temporin biological activity. Therefore, the term "fragment" is not unclear and does not refer to a single amino acid.

1.5

It is asserted that claims 1-11 and 13 are not supported by the present application.

Applicants respectfully disagree.

There is teaching throughout the present specification how to make variant peptides, and to test those peptides for dermaseptin and temporin biological activity. For example see page 8, line 12-page 10 line 4; page 11 line 1-page 12; page 17 line 12-page 21 line 10. Therefore, claims 1-11 and 13 are supported by the present application.

2.

The boxes of Figure 1 are explained on substitute page 6.

Conclusion

For the foregoing reasons, the objections stated in the Written Opinion should be withdrawn. And a favorable International Preliminary Examination Report is solicited.

Respectfully submitted,

Sim & McBurney

Per: ORIGINAL SIGNED BY PATRICIA A. RAE

Patricia A. Rae (Dr.)

PAR:sma
Encl.

SEQ IDs: 33 shows the nucleic acid sequence encoding $MSRA_3$.

SEQ ID: 34 shows the amino acid sequence of $MSRA_3$.

SEQ IDs: 35-38 show the oligos used to generate the nucleic acid sequence encoding $MSRA_3$.

SEQ IDs: 39-41 show the amino acid sequences of various N-terminal extension sequences.

Brief Description of the Figures

Figure 1 is a graph that shows the results from assays that tested the resistance of transgenic potato tubers to soft rot. Discs prepared from tubers of *Desiree* control and transgenic plants expressing Demaseptin B (sample Nos. D1, D2, D6, D10) or Temporin A (sample Nos. T1, T2, T3) were infected with *E. carotovora* (black boxes) or left uninfected (white boxes). After 6 days at RT, rotted tissue was gently removed from the discs and the sensitivity/resistance to *E. carotovora* was expressed as the loss of weight of tuber tissue.

Figure 2 is a graph that shows the bactericidal effect of the peptides $MSRA_2$ (Dermaseptin B) and $MSRA_3$ (Temporin A) on *E. coli*. The cell cultures were incubated at room temperature in the presence of indicated concentration of Dermaseptin B (DSB; 7 μg/ml, 30 μg/ml, and 75 μg/ml), Temporin A (TA; 75 μg/ml, 133 μg/ml, 200 μg/ml) and a combination of Temporin A and Dermaseptin B (133 μg/ml Temporin A and 30 μg/ml Dermaseptin B) for 4 hours, diluted and plated on LB plates. After overnight incubation at 37°C, the colonies were counted and the survival of bacteria was scored.

Figure 3 is a graph that shows the bactericidal effect of the peptides $MSRA_2$ (Dermaseptin B) and $MSRA_3$ (Temporin A) on *E. carotovora*. The cell cultures were incubated at room temperature in the presence of indicated concentration of Dermaseptin B (DSB; 23 μg/ml, 45 μg/ml) or Temporin A (TA; 67 μg/ml, 133 μg/ml) for 4 hours, diluted and plated on LB plates. After overnight incubation at 28°C, the colonies were counted and the survival of bacteria was scored.

I. Definitions

D rmaseptin: As used herein, the term "dermaseptin" refers to any member of the family of naturally occurring cationic peptides termed dermaseptins,

11. A transgenic plant according to claim 8 wherein the peptid further comprises an anionic pro-region peptide operably linked to the N-terminus of the peptide.

12. A transgenic plant comprising a recombinant nucleic acid molecule encoding a peptide comprising an amino acid sequence selected from the group consisting of SEQ IDs: 28 and 34.

13. A method of producing a biologically active cationic peptide comprising:

providing a transgenic plant according to claim 1; and

isolating at least one biologically active cationic peptide from the plant.

14. The method of claim 13, wherein the cationic peptide is selected from the group consisting of the dermaseptins set forth in SEQ ID NOs 3-14, and the temporins set forth in SEQ ID NOs 17-26.